Biological and molecular aspects of *Sodalis glossinidius*

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Declaration

I declare that the research described within this thesis is my own work and that this thesis is my own composition

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Contents

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Abstract

Glossina spp. harbour three symbiotic bacteria: *Wigglesworthia glossinidia,* on which the fly depends for the production of essential B vitamins; *Wolbachia* spp., which may be involved in causing reproductive anomalies and *Sodalis glossinidius*, which as yet has not been shown to have any positive input on the fly but may be involved in susceptibility to trypanosome infection. The current work focuses on the biological and molecular aspects of *S. glossinidius* but also examines the prevalence of *Wolbachia* spp. in wild tsetse populations.

One of the current theories is that the number of *S. glossinidius* present when the tsetse takes its first blood meal may be an important factor in determining susceptibility to infection. Therefore, the quantification of the number of *S.glossinidius* bacteria present in tsetse may provide an insight into this mechanism. Utilising quantitative PCR, the levels of *S. glossinidius* were quantified and it was found that the bacterial population size is dynamic over the developmental course of the fly.

S. glossinidius is one of the few insect symbionts that can be cultured *in vitro.* The *in vitro* culture of this bacterium has been optimised in this work, reducing the time taken for isolation of *S. glossinidius* by seven days. The growth pattern of *S. glossinidius* was measured and used to evaluate the effect of stress conditions on the bacterium. It was found that iron was an essential nutrient for the growth of this bacterium and the growth of *S. glossinidius* was found to be inhibited in iron-deficient medium. *S. glossinidius* was also found to synthesise siderophores in response to these growth conditions.

The prevalence of *S. glossinidius* was analysed in wild flies, with significant differences being found both between different tsetse species and between individual species sampled from different countries. Laboratory colonies, however, exhibit *S. glossinidius* prevalences of 100% which this work has shown may be due to the horizontal transmission through urophagous behaviour during blood meals. The prevalence of *Wolbachia* spp. was seen to change over time, with the infection sweeping into one population sampled. This may suggest that *Wolbachia* spp. causes reproductive abnormalities in the tsetse as it is known to do in other insects.

It is concluded that the horizontal transmission of *S. glossinidius* is likely to account for the ubiquity of this symbiont in laboratory colonies of tsetse and may have adversely influenced the studies performed to date on this bacterium.

List of Figures

List of Graphs

List of Tables

List of Equations

List of Boxes

Abbreviations used

BUT 135	Buteba 135
CAS	Chrome asurol S
CFU	Colony forming unit
CI	Cytoplasmic incompatibility
CNS	Central nervous system
CTAB	Cetyltrimethylammonium bromide
DALY	Disability adjusted life years
DAPI	4',6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assays
FCS	Foetal calf serum
IFA	Immunofluorescent antisera preparation
MMI	Mitsuhashi and Maramorosch medium
NTA	Nitrilotriacetic acid trisodium salt
OD	Optical density
ORF	Open reading frame
PAR	Pea aphid rickettsia
PASS	Pea aphid secondary symbiont
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RLO	Rickettsia-like organism
SLS	Scientific Laboratory Supplies
SOPE	*Sitophilus oryzae* primary endosymbiont

SZPE	*Sitophilus zeamis* primary endosymbiont
TTSS	Type three secretion system
wsp	Wolbachia surface protein

Chapter 1

Introduction

1 Introduction

> "To many, no doubt, such speculations may appear too fantastic for present mention in polite biological society; nevertheless it is within the range of possibility that they may some day call for some serious consideration" (Wilson, 1925) in (Sapp, 1994)

1.1 Defining symbiosis

> "Defining symbiosis has almost become a kind of life science cliché, an act of verbal and often verbose masochism." (Zook, 1998)

The word 'symbiosis' derives from the Greek word for living together, although no definition of its current usage is universally accepted. Although it is commonly attributed to Anton de Bary, the term was in fact first coined by Albert Bernhard Frank in 1877. He sought to find a neutral term to describe associations between phylogenetically distinct organisms that would cover the wide ranges, from facultative to essential and one-sided to beneficial, for which the term 'parasitism' held too many negative connotations.

> "We must bring all the cases where two different species live on or in one another under a comprehensive concept which does not consider the role which the two individuals play but is based on the mere coexistence and for which the term Symbiosis [*Symbiotismus*] is to be recommended." (Frank, 1877) in (Sapp, 1994)

A year later de Bary first used the term symbiosis and defined it as "the living together of unlike named organisms". Again, this definition encompassed a variety of interactions that range along a continuum from parasitic to beneficial.

As a concept, symbiosis was slow to gain acceptance within the scientific community. Many considered that any such interaction between two disparate organisms must be parasitic in nature and the idea of "a useful and invigorating parasitism" was both impossible and absurd (Crombie, 1874) in (Sapp, 1994).

Using de Bary's definition, symbiotic interactions cover a broad and dynamic range from mutualism to parasitism, where a mutualistic relationship is to the benefit of both parties and a parasitic one benefits one organism at the cost of the other. However, this is the area over which many scientists disagree. The term symbiosis has come to be used as synonymous with mutualism by some. For instance, Richard Dawkins states:

> "A relationship of mutual benefit between members of different species is called mutualism or symbiosis." (Dawkins, 1989)

Parasitism is often treated as entirely distinct from symbiosis. This view regards the cost-benefit interaction between organisms as fundamental to the classification of their relationship. In contrast, taking the intimate and prolonged association between two organisms as the defining factor of their relationship, parasitism becomes considered alongside mutualism as a symbiosis.

In certain circumstances, some organisms are able to transition from symbiotic to pathogenic lifestyles. One such organism is *Photorhabdus luminescens*, a symbiotic bacterium of the nematode *Heterorhabditis bacteriophora*. The nematode transmits *P. luminescens* into the body cavity of an insect host, where the microorganism produces numerous toxins, which are highly pathogenic to the insect. (reviewed by (ffrench-Constant *et al.*, 2003). Such a dual lifestyle lends further complication to the boundaries of definitions of these interactions.

A more recent definition of symbiosis holds that the acquisition and maintenance of the microorganism will result in novel structures or metabolism (Zook, 1998). Whilst affirming the importance of longevity in the symbiotic relationship, this definition makes no presumption as to the beneficial nature of the relationship. Measuring the cost-benefit interaction between two organisms can be a difficult process. The time range and conditions under which the interaction is assessed must be carefully considered lest they distort the results of the study (Dillon & Dillon, 2004).

In contrast to the broad range of interactions covered by the term symbiosis, endosymbiosis maintains a far narrower definition laid down by Buchner (Buchner, 1965):

> "By the term endosymbiosis we mean well-regulated and essentially undisturbed co-operative living between two differently constituted partners. It is usually a far more organized partner which shelters another within its body, and the mutual adaptation is so complete as to justify the assumption that the arrangement is useful to the host."

This definition clearly specifies that the relationship should be beneficial to both parties and, to a large extent, excludes the more labile symbioses typified by gut microbiota and facultative symbiotic bacteria. Buchner was primarily concerned with the study of mycetocyte endosymbioses in insects and in some ways, this has coloured the terms of his definition. Since then, the term endosymbiosis has come to encompass a wider variety of interactions including all those symbioses which are intra- or extracellular within their hosts. Extracellular symbionts may inhabit internal cavities of the host, such as the gut, or reside between cells in host tissue. This latter example is typified by the mutualistic symbiosis between mycorrhizas and plants and may be termed intercellular symbiosis (Strack *et al.*, 2003).

The influence of symbiotic associations on the evolution of life is thought to have been profound. There are many theories that controversially place symbiotic microorganisms as the forerunner of both mitochondria and chloroplasts, as well as other cellular organelles (Margulis, 1971; Lopez-Garcia & Moreira, 1999; Margulis *et al.*, 2000; McFall-Ngai, 2001). The endosymbiotic theory of chloroplast evolution dates back to 1905, when Mereschkowsky's hypothesis stated that plastids are reduced forms of cyanobacteria acting as "little workers, green slaves" within the cell (Dyall *et al.*, 2004).

Prokaryotic organisms possess a broad range of metabolic capabilities, in contrast to the relatively limited range in eukaryotes. The recruitment of symbiotic microorganisms has given plants and animals access to these metabolic properties and enabled them to exploit new ecological niches and survive on otherwise nutritionally deficient or even toxic diets (Dillon & Dillon, 2004). In this way, symbiosis has acted as an "evolutionary strategy" for eukaryotes, allowing them to tap a greater variety of nutritional resources (Latorre *et al.*, 2005).

The present work concentrates on the nature of the endosymbiosis of the tsetse fly (*Glossina* spp.) with one species of its common microbiota, *Sodalis glossinidius*, whilst also examining the occurrence of *Wolbachia* spp. infections in wild tsetse populations. The nature, dynamics and maintenance of the symbiotic relationship between *Glossina* spp. and *S. glossinidius* are considered here.

For the purposes of this work, symbiosis has been defined as the close, prolonged and maintained association between members of different species that results in novel structures or metabolism. Host-symbiont interactions are defined as being either beneficial or neutral in net effect, thus excluding parasitic relationships.

1.2 Endosymbiotic associations with insects

> "Probably the greatest impact of bacteria upon hosts can be found in the phylum Arthropoda." (Dasch *et al.*, 1984)

Arthropods have formed symbiotic relationships with a broad range of microorganisms, including bacteria, fungi and viruses. An estimated 10% of known insect species play host to a melange of non-parasitic microorganisms, some of which show extreme host-specificity (Douglas, 1989). These microorganisms have a variety of functions; some are harmful to their host, some neutral and others beneficial, even essential to the arthropod.

The associations in arthropod-microorganism symbioses vary greatly in their intimacy. Some species of termites, ants and beetles have formed symbiotic relationships with various species of fungi whereby they act as 'farmers', tending to the fungal growth in order to feed their colony (Mueller & Gerardo, 2002). At the other end of the spectrum are endosymbiotic bacteria that live inside the cells of their host.

The endosymbionts of Arthropods may be divided into three categories: primary symbionts, secondary symbionts and gut microflora. However, the terminology for the first two categories is not consistent within the literature. Primary symbionts may also be referred to as mycetocyte or nutritional symbionts (Douglas, 1989). These terms reflect the defining characteristics of these interactions. Secondary symbionts have also been termed facultative symbionts or accessory bacteria (Darby & Douglas, 2003). This is indicative of the use of the narrower definition of

symbiosis as being equivalent to mutualism, since despite living in close and prolonged association with their host, these bacteria do not infect 100% of their host population and have unknown, and possibly non-beneficial roles in the relationship.

1.2.1 Primary symbionts

Primary symbionts are found in specialised host cells, called mycetocytes, which may be located in various sites around the insect's body, such as the fat body, haemocoel, and the midgut (Douglas, 1989). There are three main characteristics that are commonly shared by these symbionts. Firstly, they possess metabolic capabilities that are absent in the host, thereby performing an essential function in supplementing the host's deficient diet. Secondly, the association of the primary symbiont with its host is absolutely necessary for the survival of both organisms as the symbiont is so specialised to growth in its specific habitat that it is usually unculturable *in vitro* and the host suffers malnutrition in the absence of its symbiont. Thirdly, primary symbionts are vertically transmitted by the host to its offspring, thus ensuring the continuation of the symbiosis in the next generation (Douglas, 1989).

It has long been noted that most insects that have primary, mycetocyte symbionts also have nutritionally depauperate diets (Buchner, 1965; Douglas, 1989). For example, vertebrate blood lacks B vitamins, essential for normal growth and reproduction, plant sap has an imbalance of amino acids and wood has little nutritional value. That so many insects survive and thrive on these apparently inadequate diets is likely due to the populations of microorganisms that they play host to.

The primary endosymbiont of aphids (Homoptera, *Aphididae*), *Buchnera* spp. is one of the most extensively studied symbionts of insects. The association is considered to be intimately mutualistic (Fukatsu *et al.*, 2000) as the bacteria are unable to be

cultured outside their host and the host becomes sterile or even dies when 'cured' of the symbiont infection (Griffiths & Beck, 1973).

1.2.2 Secondary symbionts

In contrast to primary symbionts, accessory bacteria are not essential to the survival or fecundity of their host, although the host is often the only habitat in which the symbiont is found. The function of accessory bacteria has not yet been determined in many cases, indeed the relationship may appear to be more like commensalism with the insect gaining little or no benefit from the bacteria's presence. The association between accessory bacteria and their hosts is also often labile, with uninfected individuals found with high frequency in wild populations (Welburn & Gibson, 1989; Maudlin *et al.*, 1990; Cheng & Aksoy, 1999; Fukatsu *et al.*, 2000).

Secondary symbionts are thought, by some, to be examples of a preliminary stage in the evolution of primary symbiosis (Klasson & Andersson, 2004; Latorre *et al.*, 2005). Their wider tropism within the host insect may be seen as a form of maladaption, resulting from a relatively short period of coevolution of the two organisms (Fukatsu, 2001; Fukatsu *et al.*, 2000; Sandstrom *et al.*, 2001).

1.2.3 Gut microbiota

There is wide variation in the numbers of microbes that colonise different species of insects and, whilst some arthropods, for example many aphids, have sterile digestive tracts, it is likely that most insect species harbour a community of microorganisms that outnumbers even their own cells (Douglas, 1989; Dillon & Dillon, 2004).

Complex microflora may include maternally transmitted and environmentally acquired microorganisms and there may be a rapid turnover of microorganisms (Conte, 1997; Dillon & Charnley, 2002). This turnover necessitates the careful study of the microbiota of the gut to determine which microorganisms engage in a prolonged relationship with their host and may therefore be considered to be symbiotic (Dillon & Dillon, 2004).

Gut microbiota of insects are often acquired through the insect's diet and reflect the local environment (Dillon & Charnley, 2002). The gut symbionts of the Heteroptera (true bugs) are generally maternally inherited through contamination of the egg shell with symbiont-containing excreta (Fukatsu & Hosokawa, 2002).

In some insects, gut symbionts play an important role in digestion and are therefore necessary for the fitness of the host (Slaytor *et al.*, 1997; Lilburn *et al.*, 2001). These nutritional contributions may include the production of digestive enzymes or synthesis of vitamins – benefits which could improve the efficiency of host digestion and widen the range of potential host diets respectively (Dillon & Dillon, 2004). In common with other organisms such as mammals, insect gut microbiota can also help protect their host from infection with pathogenic microorganisms, in a manner known as colonisation resistance (Dillon & Charnley, 2002; Nakabachi *et al.*, 2003). Here, the growth of pathogenic microorganisms is inhibited by the presence of symbiotic or commensal bacteria.

1.3 Genome characteristics and evolution of endosymbioses

1.3.1 Genome degradation

The DNA sequence evolution of endosymbionts is characterised by a lack of recombination, elevated mutational rates and biases and the fixation of deleterious mutations by random genetic drift. Endosymbiont populations are strictly asexual, preventing any recovery of wild type genotypes by recombination. This is a process by which the number of deleterious mutations increases irreversibly in asexual populations due to their inability to restore the fittest genotype by recombination. The strictly asexual nature of endosymbionts may result from a number of factors. These bacteria have lost certain gene elements (such as transposons and prophages), as well as genes with recombinational functions (such as *recA* and *recF*) (Dale *et al.*, 2003; Ochman & Davalos, 2006). Some endosymbionts are also sequestered in specialised host cells, which limits the contact that they might otherwise have with genetically distinct strains.

The fixation of deleterious mutations in endosymbionts is also caused by the 'bottlenecks' imposed on the population as a result of their strict vertical mode of transmission. The number of bacteria passed to individual offspring varies by host species but is necessarily only a small fraction of the original population. Successive bottlenecks make the effective population size orders of magnitude smaller than that of free-living bacteria. This serves to elevate the rate of fixation of deleterious mutations through genetic drift and in time could have a negative effect on the fitness of both the symbiont and its host.

The genomes of obligate endosymbionts are often far smaller that those of their free-living counterparts. This is in common with intracellular bacterial pathogens with the smallest known pathogen genome measuring 580 kbp (*Mycoplasma genitalium*)

in comparison with the smallest known endosymbiont genome at 450 kbp (*Buchnera aphidicola*). For comparison, *Escherichia coli* K-12 has a genome measuring 4.6 Mb (Blattner *et al.*, 1997). The genome of the majority of bacteria is composed of coding DNA so the dramatic losses seen in endosymbionts are primarily through the loss of genes involved in metabolic function and physiological capacities. The gradual reduction of biosynthetic capabilities of the symbiont may impose severe constraints on the evolutionary potential of these bacteria and may help to account for the unculturability of this class of microorganisms in general.

The genomes of obligate endosymbionts are also characterised by their unusually low G+C contents. Free-living Proteobacteria, such as *E. coli* have a G+C content of approximately 50%, but endosymbionts tend to have more A+T rich genomes with G+C contents as low as 35% (Muto & Osawa, 1987; Heddi *et al.*, 1998). The exact reason for this apparent A+T accumulation in many endosymbionts and other intracellular bacteria is as yet undetermined, but may result from directional mutational pressure (Heddi *et al.*, 1998).

An exception to the extremely small genome size of primary endosymbionts is the primary endosymbiont of the weevil, *Sitophilus oryzae* (SOPE). This endosymbiont has a relatively large genome (3.0 Mb) (Charles *et al.*, 1997) and a higher G+C content (54%) (Heddi *et al.*, 1998) than its closely related counterparts (4-5 Mb and 39.5% on average respectively) (Heddi *et al.*, 2001). In fact SOPE is not an obligate endosymbiont but forms a facultative relationship with its host and so may represent a recently established endosymbiosis (Heddi *et al.*, 1998).

Host-level selection is displayed in the range of genes that have not been lost from endosymbiotic genomes. Genes for specific biosynthetic functions that are essential to the host have been retained in both *Wigglesworthia glossinidia* (Chen *et al.*, 1999) and *Buchnera aphidicola* , the primary endosymbionts of *Glossina* spp. and the aphid

Acyrthosiphon pisum respectively. Genomic analysis of other endosymbionts with as yet unknown functions for the host may help elucidate their role in the symbiosis by analysis of the genes that are retained by the bacterium. It has also been shown that endosymbionts may rely on their host for specific nutrients. In the case of *B. aphidicola* the endosymbiont relies on its aphid host for the supply of non-essential amino acids, coenzyme A and perhaps polysaccharides (Shigenobu *et al.*, 2000). The co-dependence of this host-symbiont relationship is evidenced by the "complementarity of gene repertoire between host and symbiont" (Shigenobu *et al.*, 2000).

1.3.2 Symbiosis islands

Genomic islands are approximately 10 – 100 kb blocks of genes encoding related functions that may be horizontally transferred and inserted into recipient genomes (Hacker & Carniel, 2001). Pathogenicity islands, which are collections of genes found in distinct locations of the genome that confer pathogenic phenotypes on the microorganisms that contain them and are absent from non-pathogenic types, are the best known example of these genetic elements (Groisman & Ochman, 1997).

Genomic islands that confer selective advantages to their 'host' under specific environmental conditions are termed fitness islands (Preston *et al.*, 1998). Fitness islands can be considered in the light of their host's lifestyle and in this way, those islands which play a role in the interactions between a symbiotic partners is called a symbiosis island (Finan, 2002). For example, *Mesorhizobium loti*, a symbiotic bacterium of several *Lotus* species, has been found to harbour a symbiosis island encoding nitrogen fixation genes (Sullivan & Ronson, 1998).

S. glossinidius has been found to contain two putative symbiosis islands, encoded on one of the extrachromosomal elements (pSG1) of this bacterium (Darby *et al.*, 2005).

The first of these encodes genes for the production and transport of siderophores. These are low molecular weight compounds synthesised by bacteria that are used to chelate iron in environments that are deficient in this essential metal ion. Iron is often sequestered by iron-binding proteins in the host and very little, approximately only 10^{-18} M ferric iron is freely available, a limiting amount for bacterial growth (Andrews *et al.*, 2003). Many pathogenic bacteria, such as *Pseudomonas* spp. and uropathogenic *E. coli* use siderophores to enable them to overcome host iron-binding proteins and thrive in an otherwise iron-limited environment (Budzikiewicz, 2001; Bauer *et al.*, 2002). The second putative symbiosis island carried on pSG1 is thought to encode effectors, toxins, haemolysins, and protease fitness factors (Darby *et al.*, 2005).

Three other symbiosis islands have been identified on the *S. glossinidius* chromosome, each encoding a type III secretion system (TTSS) (Dale *et al.*, 2005; Toh *et al.*, 2006). TTSSs are conserved structures comprised of a syringe-like mechanism by which a bacterium may 'inject' effector proteins into another cell to assist in host cell invasion (Dale & Welburn, 2001; Deng *et al.*, 2004). TTSSs are commonly associated with pathogenicity islands carried by animal and plant pathogens such as *Salmonella* spp., *Yersinia pestis* and *Erwinia* spp. and in these cases are considered virulence factors (Dale *et al.*, 2002).

Two of the TTSSs of *S. glossinidius* have been shown to play a role in host cell entry and post invasion processes in *in vitro* cell culture studies (Dale *et al.*, 2002; Dale *et al.*, 2005). TTSS-deficient mutants of *S. glossinidius* are incapable of cell invasion *in vitro* and are aposymbiotic when injected into tsetse flies suggesting that the TTSS plays an important role in the life cycle of this bacterium (Dale *et al.*, 2001). That a virulence factor should be necessary in the life cycle of a symbiotic bacterium is thought by some to add credence to the hypothesis that symbiotic bacteria have evolved from pathogenic or parasitic associations with the host organism upon the establishment of a vertical mode of transmission (Dale *et al.*, 2001). The primary

endosymbiont of *Sitophilus zeamis* (SZPE), the closest relative of *S. glossinidius*, also has genes encoding a TTSS (Heddi *et al.*, 1998; Dale *et al.*, 2002).

1.3.3 Co-evolution of endosymbionts with insect hosts

Primary endosymbionts, which are obligatory for the viability and fecundity of their hosts, often show co-evolution with their host species. This is consistent with their stable transmission from mother to offspring through many generations. Such phylogenetic congruence indicates that there has been host-symbiont cospeciation. This can often be traced back to a single infection in each host group (Moran *et al.*, 1993; Baumann *et al.*, 1995; Chen *et al.*, 1999; Clark *et al.*, 2000; Thao *et al.*, 2000; Degnan *et al.*, 2004) .

Symbiotic relationships have proved difficult to study in detail because of their complexity. Where one, or both, organisms rely on the other for vital functions such as nutrient provision, separation of the two has proved to be severely detrimental, resulting in infertility and/or reduced longevity of aposymbiotic progeny (Nogge, 1976).

In the case of *Glossina* spp. and its primary symbiont, *W. glossinidia,* aposymbiotic host fitness has been restored, to some extent, by the supplementation of the fly's diet with B vitamins (Nogge, 1981). These findings served to clarify the nature of the nutritional contribution made to tsetse by *W. glossinidia* and this has been confirmed by subsequent analysis of the genome, which has revealed that genes for B vitamin production have been retained, despite massive genome degradation (Akman *et al.*, 2002).

1.4 The tsetse fly

1.4.1 Taxonomy and geographical distribution of *Glossina* spp.

Tsetse flies belong to the Order Diptera and have been placed within their own family, the Glossinidae containing a single genus, the *Glossina*. The common name of the fly "tsetse" is derived from the noise which they make when flying. At present thirty-one species and sub-species have been identified (Table 1.1). These are further divided into three sub-genera, the Glossina (*morsitans* group), Nemorhina (*palpalis* group) and Austenina (*fusca* group), although some authors suggest that *G. austeni* should be placed in its own group, the Machadomyia (Chen *et al.*, 1999). The group names shown in brackets refer to the most common species in each of the three sub-genera.

Tsetse flies are found across sub-Saharan Africa and are divided into three geographical categories, East, Central and West African. They infest around 8.5 million km^2, representing more than 40% of the total land area of 37 of the 52 African countries (Allsopp, 2001) (Cattand *et al.*, 2001). Their populations are generally limited in the north by the Sahara and Somali deserts and in the south by the Kalahari desert (Figure 1.1).

Group	Species and subspecies
Glossina (*morsitans*)	*G. austeni* *G.longipalpis* *G.morsitans morsitans* *G.morsitans submorsitans* *G.morsitans centralis* *G. pallidipes* *G.swynnertoni*
Nemorhina (*palpalis*)	*G.caliginea* *G.fuscipes fuscipes* *G.fuscipes martini* *G. fuscipes quanzensis* *G.palpalis palpalis* *G.palpalis gambiensis* *G.pallicera pallicera* *G. pallicera newstead* *G.tachinoides*
Austenina (*fusca*)	*G. brevipalpis* *G.fuscipleuris* *G.fusca fusca* *G.fusca congolense* *G.haningtoni* *G.longipennis* *G.medicorum* *G.nashi* *G.nigrofusca hopkinsi* *G.nigrofusca nigrofusca* *G.severini* *G.schwetzi* *G.tabaniformis* *G.vanhoofi* *G.frezili*

Table 1.1: Species and subspecies of *Glossina*.

The three groups of tsetse flies, Glossina, Nemorhina and Austenina, are also generally distributed across different habitats. The Glossina group flies are adapted to more arid conditions and are found in the savannah. The Nemorhina group flies are mainly found in riverine forests in Central and West Africa. Austenina group flies are found in forests in Eastern Africa and are adapted to more humid conditions (Moloo, 1993).

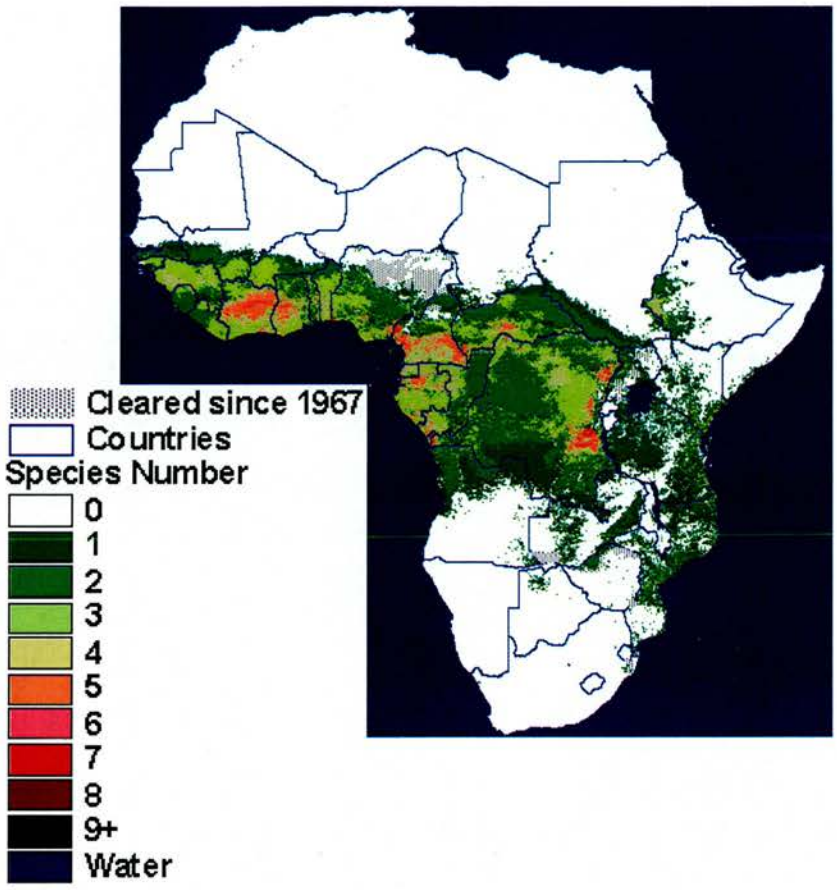

Figure 1.1: Distribution of tsetse flies across sub-Saharan Africa (Taken from the Environmental Research Group Oxford, http://ergodd.zoo.ox.ac.uk/tseweb/all_species.htm, 07/10/06).

1.4.2 Life cycle of the tsetse fly

Tsetse are unusual amongst insects (mostly r-strategists) in that they are k-strategists, reproducing by adenotrophic viviparity (Buxton, 1955). Typically, k-strategists have a relatively long life-span, produce few progeny and invest heavily in terms of energy for each offspring. This method means that a single, fertilised egg is retained within the uterus of the fly where it is supplied with nutrients from the mothers 'milk

glands'. Here the larva develops to the third larval stage before it is deposited. This larva then burrows into the ground and forms a hard puparial case around itself within an hour. An adult fly will emerge from this case around three weeks later, depending on the temperature.

Due to the huge cost in energy and raw material that is required for each pregnancy, only one larva can be produced every 7 to 12 days. A female fly may be expected to produce up to twelve offspring under optimal conditions in the laboratory, but in the wild this number is speculated to be far lower. The protected environments in which the larval and pupal stages develop (*in utero* and underground respectively) serve to minimise deaths due to predation. This fact compensates for the low birth rate of the tsetse fly.

Both sexes of tsetse fly feed exclusively on blood, which provides both their nutrient and water requirements. Vegetation is still needed for shelter, but tsetse are well adapted to survive in dry conditions.

1.5 Endosymbiosis in the tsetse fly

1.5.1 Different types of microorganisms in tsetse

Several different microorganisms may be found in the tsetse fly (see Figure 1.2). Three of these microorganisms are bacteria that are regularly associated with tsetse flies both in laboratory colonies and in wild populations. These bacteria are *W. glossinidia*, *S. glossinidius* and *Wolbachia* spp. (Dale & Welburn, 2001). The first two of these bacteria are found exclusively in *Glossina* spp. whilst *Wolbachia* spp. infect a wide range of insect species. In addition to these bacteria, three other organisms are associated with tsetse flies, although usually they are only found in wild populations. The most important of these three are members of the trypanosome family, which are the causative agents of sleeping sickness as well as causing nagana in animals (Welburn & Maudlin, 1999). The second microorganism is a baculovirus that causes ovarian anomalies, testicular degeneration and enlargement of the salivary glands of the fly (Sang *et al.*, 1999). The third is a parasitic nematode that invades the larvae of the tsetse and can be found in the haemolymph of the adult fly (Moloo, 1972).

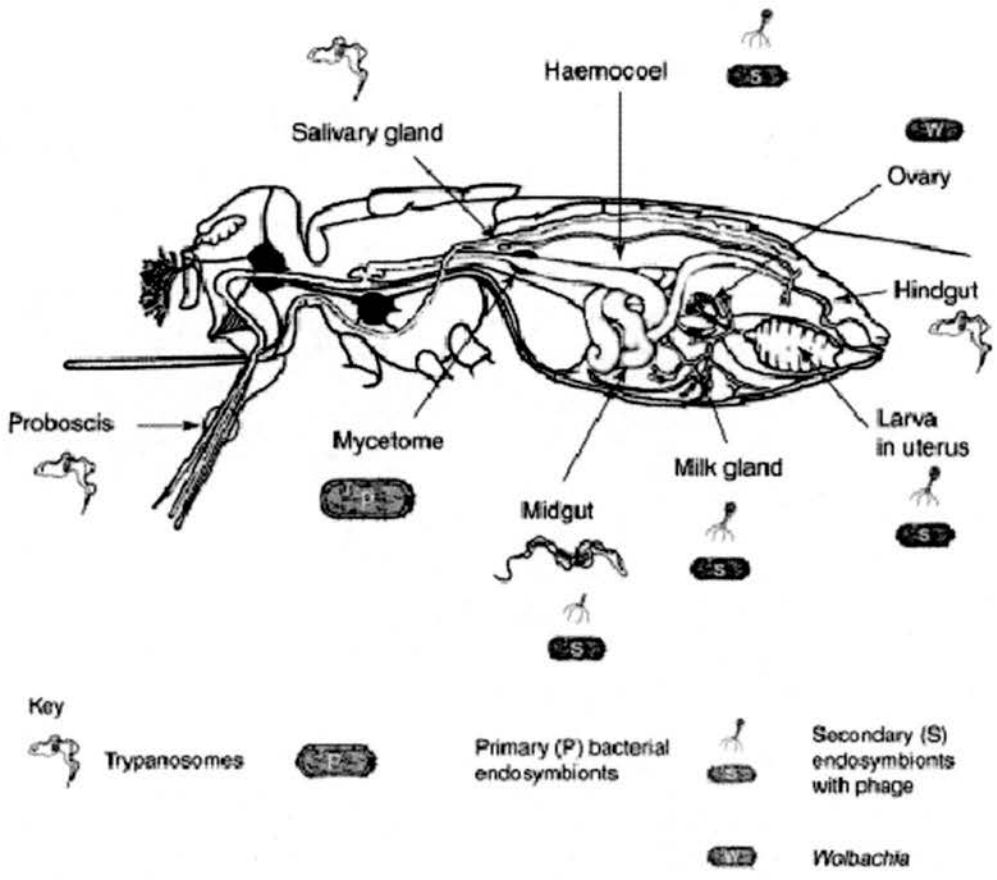

Figure 1.2: Organisms found within the tsetse fly (kindly provided by Prof. Sue Welburn)

1.5.2 *Wigglesworthia glossinidia*

W. glossinidia represents a monophyletic group within the γ-3-subdivision of Proteobacteria. It is a Gram negative rod-shaped bacterium around 8-10 μm long and 1.5-2 μm in diameter (Aksoy, 1995). *W. glossinidia* is found exclusively within specialised epithelial cells, mycetocytes, which form the mycetome in the anterior portion of the tsetse's gut.

The vertical transmission of *W. glossinidia* from mother to progeny has long been thought to take place via the maternal secretion from the milk gland, with which the developing larva is fed whilst *in utero* (Buxton, 1955). This is similar to the manner in which the primary endosymbionts of *Pupipara* are transmitted (Buxton, 1955; Buchner, 1965). This hypothesis was supported by the observation of bacteria in the lumen of milk glands by microscopy (Ma & Denlinger, 1974). Although Aksoy *et al.* (Aksoy *et al.*, 1997) found *S. glossinidius* DNA in the milk gland secretions, no *W. glossinidia* DNA was detected. However, there are inconsistencies in the literature and several publications state that milk gland secretion is the route of *W. glossinidia* transmission (Akman & Aksoy, 2001; Akman *et al.*, 2002).

In common with many other primary endosymbionts, *W. glossinidia* has proved to be unculturable *in vitro*. Despite this limitation, PCR analysis has enabled the investigation and phylogenetic analysis of unculturable endosymbionts from aphids (Rouhbakhsh *et al.*, 1994), carpenter ants (Schroder *et al.*, 1996), cockroaches (Bandi *et al.*, 1994) and tsetse flies (Cheng & Aksoy, 1999). Analysis of *W. glossinidia* 16S sequences has demonstrated that these symbionts have undergone concordant evolution with their host and the evolutionary relationships of different *Glossina* species are closely reflected in their *W. glossinidia* symbionts (Chen *et al.*, 1999) (Aksoy *et al.*, 1997).

As an obligate endosymbiont, *W. glossinidia* plays an essential role in the viability of its host. In common with other insects that have nutritionally unbalanced diets, the tsetse fly's exclusive diet of blood lacks vitamin B complex metabolites. These are synthesised by *W. glossinidia* and aposymbiotic insects can only be maintained when supplementary vitamins are included in their diet (Nogge, 1976, 1981)

1.5.3 *Sodalis glossinidius*

In common with most of the insect endosymbionts so far studied, *S. glossinidius* belongs to the γ-3-subdivision of the Proteobacteria (Aksoy *et al.*, 1997). This subdivision includes free-living bacteria and pathogens such as *E. coli* and *Yersinia* spp. Phylogenetic analysis has revealed that *S. glossinidius* is closely related to the primary endosymbionts of *Sitophilus* weevils and more distantly to *E. coli* (Moran & Mira, 2001).

1.5.3.1 Phenotype

The cells of *S. glossinidius* are non-motile, non spore-forming, filamentous, Gram negative rods that are 2-12 μm long and 1-1.5 μm in diameter and divide by septation (Dale & Maudlin, 1999). The bacterium may be cultured in *Aedes albopictus* cell culture (see Figure 1.3), axenically in enriched media or on solid media under microaerophilic conditions.

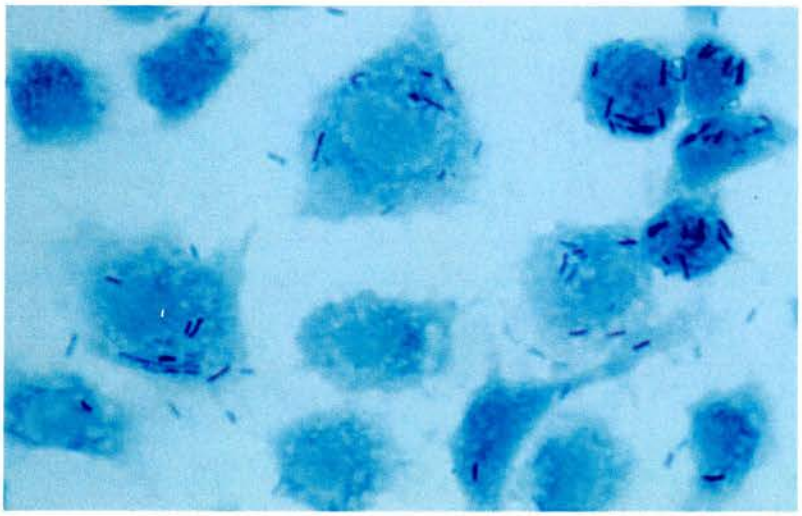

Figure 1.3: Non specific Gimenez stain detection of *Sodalis glossinidius* in association with C6/36 cells (kindly provided by Prof. Sue Welburn)

1.5.3.2 Tropism

S. glossinidius has a broad tissue tropism throughout the tsetse fly and is harboured both intra- and extracellularly, although it is mainly found in midgut tissue. The tissue tropism of *S. glossinidius* has been reported in several different species of tsetse fly (*G. m. morsitans, G. p. palpalis, G. austeni* and *G. brevipalpis*) using primers specific for one of the extrachromosomal elements of the bacterium (Cheng & Aksoy, 1999). *S. glossinidius* was mainly found in the midgut, haemolymph and milk gland of teneral flies. In *G. p. palpalis*, *S. glossinidius* was also detected in the salivary glands of teneral flies. In older flies *S. glossinidius* infections were found in an increased range of tissues including muscle, testes and fat bodies. The distribution of *S. glossinidius* in older flies has been hypothesised to represent the ability of the bacterium to replicate extracellularly and invade new tissues (Cheng & Aksoy, 1999). The detection of the bacterium in various tissues was made semi-quantitative by the classification of the intensity of the PCR-amplification product based on the ethidium bromide staining. This analysis found that *G. m. morsitans* and *G. p. palpalis* had considerably greater densities of *S. glossinidius* infection than *G. austeni* and *G. brevipalpis*, a finding in part supported by previous studies by microscopy by Moloo and Shaw. (Moloo & Shaw, 1989; Cheng & Aksoy, 1999)

1.5.3.3 Genome size

Although the size of the genome of *S. glossinidius* had been estimated, by means of contour-clamped homogeneous electric field (CHEF) gel electrophoresis to be approximately 2 Mb and the coding capability examined by hybridisation to *Escherichia coli* microarrays (Akman *et al.*, 2001), the recent publication of the genome has shown that it is more than double the original estimate and is in fact 4.17 Mb (NC_007902) (Toh *et al.*, 2006). Whereas microarray hybridisation has detected 1800 orthologues, representing around 85% of the then estimated genome, the recent analysis has found 2300 genes suggesting a high proportion of pseudogenes and a

very low coding density of only 51%. This is comparable to obligate intracellular bacteria such as *Mycobacterium leprae* (49.5%) (Cole *et al.*, 2001).

1.5.3.4 Genotype

Strains of *S. glossinidius* from different species of *Glossina* have almost identical 16S rDNA sequences. Only two out of 1100 bp were different between the rRNA sequences of *G. brevipalpis* and *G. f. fuscipes*, which represent the two most divergent subgenera (Aksoy *et al.*, 1997). This suggests that either horizontal transfer of these bacteria occurs between tsetse flies or each host species has recently acquired *S. glossinidius* independently (Aksoy, 2000; Gil *et al.*, 2002). Continued vertical transmission would have resulted in sequence divergences within the16S rRNA, as seen in *W. glossinidia* (Moran, 1996; Aksoy *et al.*, 1997; Gil *et al.*, 2002). In tsetse flies, the similarities between *S. glossinidius* from different *Glossina* species contrast with the differences between strains of *W. glossinidia*, which has coevolved with the tsetse fly for approximately 50 – 80 million years (as discussed earlier) and is limited to vertical transmission (Akman & Aksoy, 2001).

The most closely related bacteria to *S. glossinidius* are the primary endosymbionts of two species of weevil, *Sitophilus oryzae* and *Sitophilus zeamis* (SOPE and SZPE respectively). Comparative evolutionary analyses have shown that *S. glossinidius*, SZPE and SOPE diverged from a common ancestor approximately 100 million years ago (Dale *et al.*, 2003). Since this time the genomes of these bacteria have undergone degeneration and loss of gene function to different extents. Whilst *S. glossinidius* has retained intact copies of the recombinational repair genes, *recA* and *recF*, SOPE lacks a functional copy of *recF* and SZPE has functional copies of neither gene (Dale *et al.*, 2003). This may be a reflection of the different environments in which these bacteria live in their host. The two weevil endosymbionts are obligately intracellular within mycetocytes whereas *S. glossinidius* maintains both an intra- and extracellular lifestyle in many different

tissue of its host. Whilst SOPE and SZPE are in a relatively protected environment, *S. glossinidius* is likely to encounter a higher incidence of DNA damage and therefore have a greater selective pressure to retain its DNA repair enzymes.

1.5.3.5 Extrachromosomal elements

Extrachromosomal DNA is an inclusive term that refers to the non-chromosomal genetic elements found within a bacterial cell, such as plasmids or bacteriophage. *S. glossinidius* has long been recognised as having extrachromosomal elements (O'Neill *et al.*, 1993). The size of the extrachromosomal elements has been estimated, by means of digestion, to be >135 kb (Akman *et al.*, 2001). Complete sequencing has determined the size to be 139 kb (Darby *et al.*, 2005).

The extra-chromosomal DNA of *S. glossinidius* comprises four elements; pSG1 (82 kb), pSG2 (27 kb), pSG3 (c. 19 kb) and pSG4 (11 kb) (Darby *et al.*, 2005). Whilst pSG1, pSG2 and pSG4 are plasmid-like elements, pSG3 displays bacteriophage-like characteristics. With the exception of pSG3, all the extra-chromosomal elements contain a putative *RepA* gene.

The extra-chromosomal element pSG1 contains two putative symbiotic islands. The first of these contains genes with hypothetical siderophore biosynthesis and transport functions. This region has a markedly different G+C content compared to the surrounding sequences and is flanked by putative transposases, suggesting that the island is a result of horizontal gene transfer. The second putative symbiosis island contains two open reading frames (ORFs) with homology to a secreted protein of the symbiont *Photorhabdus luminescens* and a heat shock protein of the primary symbiont *Buchnera aphidicola* (Darby *et al.*, 2005).

Approximately 56% (14/25) of the ORFs from pSG3 show homology to bacteriophage sequences. However, none of the ORFs had homology with phage tail

or capsid proteins and, despite the homology that pSG3 shows to the P22-like genus of phage, its total size is only 47% that of that bacteriophage's genome (c. 39 kb) (Darby *et al.*, 2005).

1.5.3.6 Transmission

The high homology of 16S rDNA sequences (c. 0.6% divergence) within the group of *S. glossinidius* suggests either the recent, independent acquisition of the bacterium by different species of *Glossina* or transfers of *S. glossinidius* between different *Glossina* spp. hosts (Aksoy *et al.*, 1997). An example of horizontal symbiont transfer is given by *Wolbachia* spp. which is able undergo both vertical and horizontal transmission (Huigens *et al.*, 2004). Unlike *Wolbachia* spp. however, *S. glossinidius* has not been found to exist outside of the tsetse host, although this may be a consequence of some specific requirement for the establishment of this particular symbiosis.

1.5.3.7 Metabolic capabilities

Comparative analysis of *S. glossinidius* with its close relative, the weevil symbiont SOPE, has shown that these two have become specialised to accommodate their different host ecologies and have retained genes appropriate to the metabolites present in their hosts' diets (Rio *et al.*, 2003). This close association that these endosymbionts have formed with their hosts through their complementary restricted metabolic capabilities suggests that they may not readily undergo horizontal transmission to establish infections in other potential hosts (Aksoy & Rio, 2005).

1.5.4 *Wolbachia* spp.

The third bacterium that is associated with the tsetse fly is a *Wolbachia pipientis* like symbiont (O'Neill *et al.*, 1993). *Wolbachia* spp. is an intracellular α-proteobacteria and was reported for the first time in 1924 by Hertig & Wolbach having been observed in association with the ovaries of the mosquito, *Culex pipiens* (Hertig & Wolbach, 1924). In 1936 the bacterium was formally named by Hertig in honour of his colleague (Hertig, 1936). The bacteria are rickettsia-like Gram-negative rods of with dimorphic characteristics. Small irregularly formed rods, of 0.5–1.3 μm in length, and coccoid forms, of 0.25-0.5 μm in diameter, coexist with large forms, of 1.0-1.8 μm in diameter, which may contain several of the smaller forms (Stouthamer *et al.*, 1999). *Wolbachia* spp. has not been able to be cultured axenically although growth in cell culture has been achieved (O'Neill *et al.*, 1997; Dobson *et al.*, 2002; Fenollar *et al.*, 2003)

Wolbachia spp. can cause a range of reproductive abnormalities, including cytoplasmic incompatibility (CI). This condition can cause embryonic death following mating between an infected male and an uninfected female (Figure 1.4) (Bandi *et al.*, 2001). Infected females have a selective advantage over their uninfected counterparts as they can successfully mate with both infected and uninfected males, thus driving the infection through insect populations (Bandi *et al.*, 2001).

Wolbachia spp. infections are common among arthropods, with prevalence estimated at between 20 – 76% of all insect species (Stouthamer *et al.*, 1999; Jeyaprakash & Hoy, 2000; Werren & Windsor, 2000). Infections in laboratory colonies are often far higher than in wild populations and have a 100% prevalence in tsetse colonies studied (Cheng *et al.*, 2000). Heterogeneity of infection in wild populations allows an opportunity to form new, uninfected colonies with which it would be possible to properly demonstrate *Wolbachia*-mediated reproductive effects on various species of tsetse.

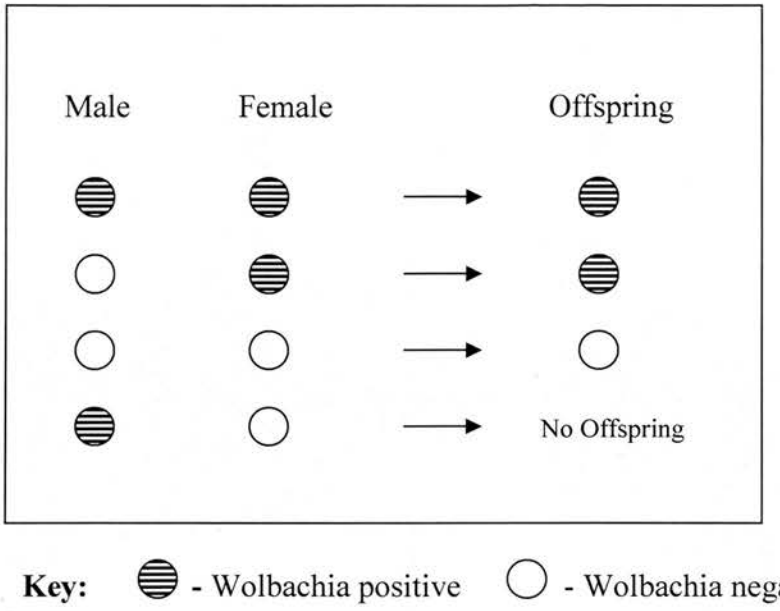

Figure 1.4: *Wolbachia* spp. –mediated cytoplasmic incompatibility in insects.

The precise effects of *Wolbachia* spp. infection in *Glossina* spp. populations are not as yet completely understood. Hybridisation experiments with tsetse have shown two types of crossing incompatibility: one attributed to nuclear genes and the other attributed to maternally inherited factors (Gooding, 2000). Crosses between *G. m. submorsitans* or *G. m. centralis* males and *G. m. morsitans* females are more compatible than the reciprocal crosses (Curtis, 1972) and it had been suggested that these incompatibilities may be caused by *Wolbachia* spp. (O'Neill *et al.*, 1993).

Wolbachia spp. infection has a prevalence of approximately 100% in laboratory colonies of tsetse and it is impossible to 'cure' the flies of this infection without also killing the essential endosymbiont, *W. glossinidia*, rendering the fly sterile (Cheng *et al.*, 2000; Aksoy, 2003).

Wolbachia spp. infections are transovarially transmitted and the bacteria have been detected in ovary and testes tissue of *Glossina* spp. by PCR. The tissue tropism of this bacteria in *G. m. morsitans*, *G. brevipalpis* and *G. austeni* has been studied using PCR amplification to detect infection (Cheng *et al.*, 2000). No *Wolbachia* spp. infection was found in laboratory colonies of *Nemorhina* group flies, but in the *Glossina* and *Austenina* groups 100% infection prevalences were measured (Cheng *et al.*, 2000). In *G. m. morsitans* and *G. brevipalpis* there was no detection of *Wolbachia* spp. infection outside of the reproductive tissue (Cheng *et al.*, 2000) although a previous study by Dobson (Dobson *et al.*, 1999) detected weak levels of *Wolbachia* spp. genomic DNA in the head, thorax and abdomen of *G. m. morsitans* males. In *G. austeni*, *Wolbachia* spp. was detected in gut, head, muscle, fat, milk gland and salivary gland tissue as well as in reproductive tissues (Cheng *et al.*, 2000).

The different tissue distribution displayed in different hosts may be due to differences in the immune regulatory mechanisms of the various host species of *Glossina*. Alternatively, differing tissue tropism may be due to inherent properties of the strains of *Wolbachia* spp. that infect these hosts. Phylogenetic analysis of these *Wolbachia* spp. strains from the three species of tsetse fly show that they represent different isolates with presumably different evolutionary histories. There may also be an influence on tissue tropism from other symbionts in the tsetse fly. Although *W. glossinidia* is restricted to the mycetocytes, *S. glossinidius* has been detected in many tissues throughout the host body. The tropism of *S. glossinidius* and *Wolbachia* spp. and their prevalence in different species of *Glossina* may give rise to competitive interaction between the two bacteria which may go on to affect their tropism and prevalence.

1.6 The African trypanosomes

African trypanosomes are protozoan parasites of the Order Kinetoplastida (Family: Trypanosomatidae; Genus: *Trypanosoma*). There are numerous species of trypanosome, with the group as a whole infecting many vertebrate genera (Barrett *et al.*, 2003). Trypanosomes are the causative agent of sleeping sickness and nagana, serious diseases of medical and veterinary importance respectively.

According to World Health Organization (WHO) statistics, approximately 60 million people across sub Saharan Africa are at risk from sleeping sickness. The disease is substantially under-reported and fewer than 10% of the population in endemic regions are screened (Odiit *et al.*, 2005). Despite the difficulties involved in making estimates in the face of such limited reporting, WHO has estimated that between 300,000 and 500,000 people are infected with sleeping sickness, resulting in approximately 100,000 deaths annually (WHO, 2006). The effects of trypanosomiasis are more apparent when the disability-adjusted life years (DALYs) are taken into consideration (Barrett *et al.*, 2003). Sleeping sickness is routinely fatal if left untreated and has recently been classified as a Category I resurgent disease by the WHO special programme for research and training in tropical diseases (WHO, 2003).

The 100% mortality of untreated sleeping sickness, alongside the social and economic impact of trypanosomiasis means that this disease is ranked third in importance, behind only malaria and schistosomiasis in terms of parasitic diseases. In some villages, in countries such as Angola and the Democratic Republic of Congo, the prevalence of human trypanosomiasis is as high as 50 %. In these provinces sleeping sickness has become one of the greatest causes of mortality, greater even than HIV/AIDS (WHO, 2006).

1.6.1 Physiology

The trypanosome is a flagellated protozoa which is approximately 10 – 120 μm long, depending on the species and lifecycle stage of the organism (see Figure 1.5). Throughout their lifecycle trypanosomes are polymorphic, transforming into bloodstream trypomastigotes, procyclic trypomastigotes, epimastigotes and metacyclic trypomastigotes. The trypanosome lives extracellularly in both the mammalian host and insect vector.

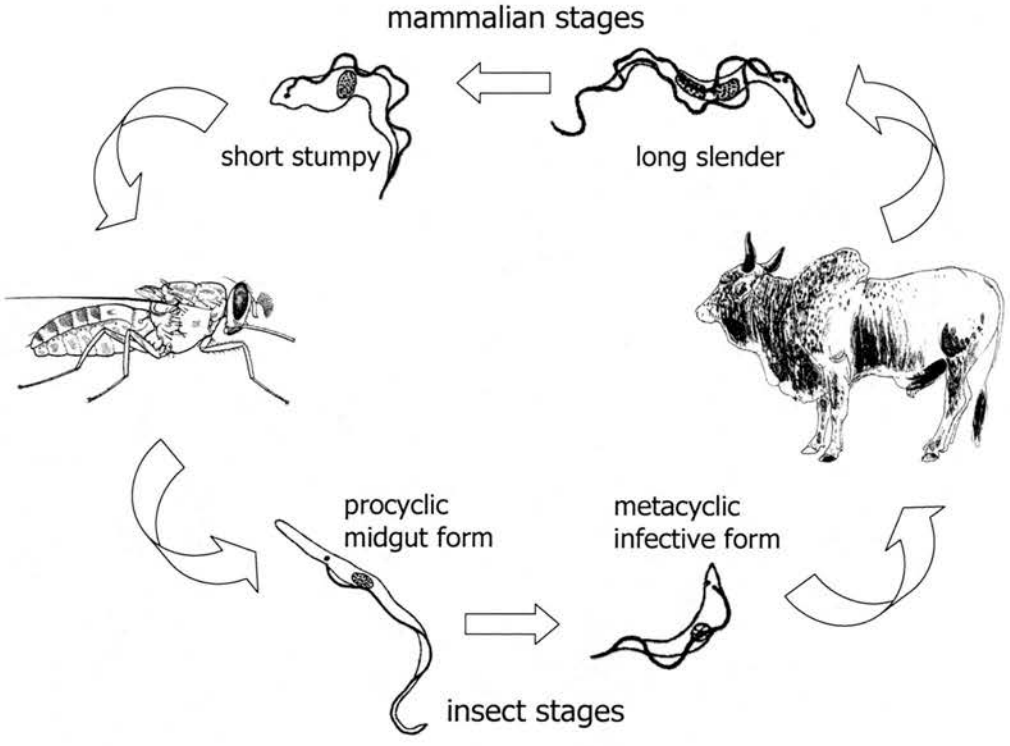

Figure 1.5: Lifecycle of *T. brucei* (kindly provided by Prof. Sue Welburn)

1.6.2 Trypanosome sub-genera

There are three sub-genera of salivarian trypanosomes that are responsible for the socially and economically important trypanosomiases in Africa. These are the *Duttonella*, *Nannomonas* and *Trypanozoon* group trypanosomes.

1.6.2.1 *Trypanozoon*

The species of trypanosome that are responsible for human sleeping sickness in Africa are *T. brucei rhodesiense* and *T. brucei gambiense*. A third subspecies, *T. brucei brucei*, causes disease in (non-primate) mammalian hosts. Although these three subspecies are morphologically indistinguishable, they give rise to very different diseases. *T. b. gambiense* characteristically gives rise to a chronic infection in humans, while *T. b. rhodesiense* causes an acute infection in humans (Welburn *et al.*, 2001). *T. b. gambiense* is localised in the West and Central Africa whilst *T. b. rhodesiense* is found in East and Southern Africa. Both subspecies are transmitted by tsetse flies in Uganda. *T. b. gambiense* is mainly transmitted by *palpalis* group tsetse flies while *T. b. rhodesiense* is mainly transmitted by *morsitans* group tsetse flies.

After ingestion the trypanosomes transform from bloodstream to procyclic forms. These remain in the midgut for 10 – 12 days before migrating to the salivary glands. Here the trypanosomes transform into mammalian infective metacyclic forms. As with *Nannomonas* group trypanosomes, the exact length of time taken for maturation is dependent upon a range of tsetse-trypanosome interactions (Dale *et al.*, 1995).

1.6.2.2 *Duttonella*

Trypanosoma vivax may be transmitted by all species of *Glossina* and as a result the parasite may be found infecting mammals throughout the tsetse belt. *T. vivax* may also be mechanically transmitted, by biting flies such as *Tabanus* spp., and so is not strictly confined to regions of tsetse infestation (Langley, 1975). *T. vivax* has a relatively short period of maturation of 5 – 13 days during which time it is confined to the proboscis of the tsetse fly (Desowitz & Fairbairn, 1955). *T. vivax* is commonly found in East Africa where it only causes mild symptoms, however, in West Africa this trypanosome can cause an acute fatal disease (Stephen, 1970).

1.6.2.3 *Nannomonas*

Trypanosoma congolense is found throughout the tsetse belt and *Glossina* spp. is the sole vector of this sub-genera. Maturation of *T. congolense* takes around 7-10 days, with the exact length of time dependent upon a range of tsetse-trypanosome interactions (Dale *et al.*, 1995). After ingestion, *T. congolense* transform from bloodstream forms into procyclic forms. These develop in the midgut for several days, after which the trypanosomes migrate to the proboscis and transform into epimastigotes. Here, *T. congolense* transforms once again into the metacyclic form and is ready to infect a new mammalian host. Chronic infection with *T. congolense* is widespread throughout East Africa in cattle.

1.6.3 Chemotherapy

1.6.3.1 Treatment of Human African Trypanosomiasis

There are four drugs that are currently licensed for the treatment of sleeping sickness (Barrett & Barrett, 2000). Pentamidine and suramin are effective in the early stages

of the disease, before CNS involvement, while melarsoprol and eflornithine are used against late-stage disease although the latter is only effective against *T. b. gambiense*. All four of the drugs may have serious side effects. The drug with the worst side effects is melarsoprol, an arsenic derivative which causes a reactive encephalopathy in approximately 20% of treated patients. As a result of this side effect up to 10% of patients die from treatment with melarsoprol (Keiser *et al.*, 2001).

Chemoprophylaxis is not used in sleeping sickness due to the toxicity of the available drugs. Vaccine research has been seriously hampered by the antigenic variation displayed by the trypanosome, although recent studies into alternative targets, such as microtubule-associated protein, are proving more hopeful (Rasooly & Balaban, 2004).

1.6.3.2 Treatment of veterinary trypanosomiasis

The drugs available for the treatment of animal trypanosomiasis include homidium chloride, ethidium bromide, suramin, diminazene aceturate and quinapyramine sulphate. Chemoprohylaxis is also appropriate for animal trypanosomiasis, using isometamidium chloride which is able to provide six months of protection from *T. congolense* (Geerts *et al.*, 1999).

1.6.4 Control methods

The uncommon reproductive strategy of the tsetse fly makes it particularly sensitive to increases in both pupal and adult mortality rates. As k-strategists, tsetse invest a relatively large amount of energy into each offspring and rely on high survival rates to maintain their population numbers. The increase of pupal and adult mortality rates using control methods such as traps, insecticides and sterile insect techniques can

cause a profound reduction in the reproductive rate of tsetse (Schofield & Maudlin, 2001).

The control of insect pests and disease vectors in general is becoming ever more complicated, partly due to the emergence of insecticide resistance but also because of other factors such as climate change and governmental policy. This has made the investigation of new methods of control increasingly important.

1.6.4.1 Vegetation clearance

The clearance of vegetation to deprive tsetse flies of their habitat was one of the first control methods used. It was first implemented on a large scale in the 1920's (Swynnerton, 1921) and was extremely effective as a means of controlling tsetse fly populations. In addition to vegetation clearance, wild mammals that offered a potential trypanosome reservoir were destroyed. Due to the enormous environmental and ecological destruction caused by these methods, they are no longer used as prime strategies for tsetse control.

1.6.4.2 Insect traps

Traps have been used in the control of tsetse fly populations since the 1930's (Harris, 1932; Harris). Their efficacy has increased over time, owing to the inclusion of olfactory, visual and auditory baits. The combination of traps with insecticides such as synthetic pyrethroids has been termed "removal trapping" (Day & Sjogren, 1994).

1.6.4.3 Insecticide spraying

During the 1950's and 1960's, organochlorine insecticides such as dichlorodiphenyl trichloroethylene (DDT) were used extensively in tsetse control (Wilson, 1953;

Glover *et al.*, 1960). This class of insecticides was withdrawn from use in developed countries in the 1970's due to environmental and public health problems resulting from their toxic and teratogenic properties. In spite of this, organochlorine insecticides were still being used for the control of tsetse populations until the 1990's (Wiktelius & Edwards, 1997).

In more recent times, synthetic pyrethroids and organophosphates have been used. Insecticides may be sprayed aerially or at ground level, selectively targeting the lower part of trees where resting flies are preferentially found (Barrett *et al.*, 2003). Insecticides have also been used in conjunction with traps and with 'live bait'. In this case, insecticides such as deltamethrin are used as either 'pour-on' (Leak *et al.*, 1995), spot-on (Gouteux *et al.*, 1996) or dip formulations for use on cattle. These strategies have been successful in significantly reducing the prevalence of trypanosome infection in cattle and lowering the population of tsetse flies (Okello-Onen *et al.*, 1994; Okiria *et al.*, 2002)

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1.6.4.4 Sterile insect technique

Sterile insect technique (SIT) involves the irradiation of male insects in order to make them sterile and their subsequent release into the wild population and has been used successfully to eradicate screw worm in North and Central America (Galvin & Wyss, 1996). The majority of female insects mate only once and then use stored sperm to fertilise their eggs. Therefore mating with a sterilized male will result in the inability to produce offspring. The release of sterile male flies on the small island of Zanzibar contributed to the complete eradication of *G. austeni* (Vreysen *et al.*, 2000). This success has prompted great hopes to use this technique more widely.

1.6.4.5 Exploitation of endosymbionts in disease control

There are several examples of the transformation of insect cells to express anti-pathogen gene products (Durvasula *et al.*, 1999; Beaty, 2000; Kokoza *et al.*, 2000). This is performed by microinjecting transposable elements on plasmids into insect eggs (Aksoy *et al.*, 2001). This method is not possible in tsetse flies because of their viviparous reproduction. Thus other methods must be found to apply transgenesis to tsetse flies. There is interest in the use of endosymbiotic bacteria as targets for genetic modification, as the transformation of culturable symbionts is less complex than that of eukaryotic insect cells (Aksoy *et al.*, 2001).

Endosymbionts are sometimes found in close association with pathogenic microorganisms within the insect vector, making genes expressed by transgenic symbionts more likely to be able to act on the pathogen (Aksoy *et al.*, 2001). This is the case in *Glossina* spp. that are infected with both *S. glossinidius* and *Trypanosoma* spp., as both organisms can be found in the midgut of the tsetse fly. As *S. glossinidius* is also easily culturable it makes trypanosomiasis an ideal model for the application of paratransgenic transformation. Paratransgenic transformation is the manipulation of the genome of a symbiotic microorganism to enable the control of disease vector in the host, amongst other objectives.

Symbiotic bacteria may be used in their host insects to introduce and express antiparasitic genes, drugs or vaccines (Hooper & Gordon, 2001). These products would be potentially capable of interfering with the pathogen's viability, development or transmission (Aksoy, 2000). Monoclonal antibodies have been identified that have parasite-transmission blocking capabilities and these have been expressed in *Rhodococcus rhodnii*, an endosymbiont of the triatome reduviid bug *Rhodnius prolixus* (Durvasula *et al.*, 1999). Multiple transmission-blocking antibodies may be used simultaneously in order to prevent the development of

resistance by the trypanosome (Aksoy, 2003). Insect immunity genes that naturally confer resistance to pathogens may also be exploited. This approach has already proved viable in *R. rhodnii* and in *S. glossinidius* (Durvasula *et al.*, 1997; Aksoy, 2003). There are obvious restrictions in that the expressed gene product must have greater toxicity for the causative agent of disease than to either the insect or the bacterial symbiont.

The reduviid bug *R. prolixus* is the vector of Chagas disease. The causative agent of this disease is *Trypanosoma cruzi*, which is carried in the hindgut of the insect (Conte, 1997). Recently *R. rhodnii* was genetically altered to express the gene for cecropin A (Durvasula *et al.*, 1997). This is a naturally occurring, pore-forming peptide that is a part of normal inducible humoral immunity in insects. The expression of this peptide was found to significantly reduce the number of trypanosomes in the gut of the paratransgenic insect (Durvasula *et al.*, 1997). This technique has now been refined to increase the construct stability by utilising an integrative shuttle plasmid to transform *R. rhodnii* (Dotson *et al.*, 2003). This inserts the foreign genes into the bacterial chromosome, thus preventing the eventual loss of non-integrative plasmids that was observed in previous studies (Dotson *et al.*, 2003).

Another approach has been to transform *R. rhodnii* to express and secrete a single-chain antibody gene product which binds progesterone (Durvasula *et al.*, 1999). Insects do not have an immunoglobulin-mediated immune response and so the expression of this antibody fragment is intended to serve as a model system which will facilitate the expression of functional, anti-trypanosomal antibodies. This technique has also been used in the malaria-transmitting mosquito, *Anopheles stephensi* (Yoshida *et al.*, 2001). *E. coli* was transformed with a gene encoding a single-chain antibody fragment fused to a synthetic peptide that resembles cecropin B (which is similar in function to cecropin A). Mosquitoes fed on the transformed bacteria showed inhibited development of the *Plasmodium* parasites.

S. glossinidius has also been successfully transformed. The marker gene, green fluorescent protein (GFP) has been introduced, is transmitted to and expressed in symbionts of F_1 and F_2 tsetse flies (Cheng & Aksoy, 1999). Potential anti-trypanosomal products for expression through this system include concanavalin A and *Glossina* attacin (GmAtt1). The former is a lectin which has previously been shown to have trypanocidal properties whilst the latter is an immune effector molecule of tsetse flies that has antibacterial and anti-trypanosomal properties (Welburn *et al.*, 1996; Hu & Aksoy, 2005). GmAtt1 has been shown to adversely affect trypanosome development *in vitro* (Hu & Aksoy, 2005). Another target could be the chitinase gene of *S. glossinidius* which are thought to indirectly increase the susceptibility of tsetse flies to trypanosome infection (Dale & Welburn, 2001). Chitinase-deficient mutants of *S. glossinidius* may lose the ability to increase the vector competence of the tsetse fly. However, *S. glossinidius* chitinase-deficient mutants have been found to be unable to infect tsetse flies (Dale & Welburn, 2001). This shows that modification, rather than elimination, of *N*-acetyl-D-glucosamine metabolism would be necessary to modulate the effect of this process on the susceptibility of tsetse flies to trypanosome infection.

In order to apply transgenesis in the field, ways to drive the transformed bacteria into and through the insect population must be found. *R. rhodnii* has been successfully introduced into *R. prolixus* through the ingestion of artificial faeces, composed of India ink and live bacterial cultures in a simulated field environment (Beard *et al.*, 1998). The resulting formulation resembles faecal droplets and is actively probed by *R. prolixus*, which becomes infected with the transformed bacteria (Beard *et al.*, 1998). This formulation could be applied to the framework of houses, theoretically preventing the domestic transmission of Chagas disease. This approach can not be utilised in the control of sleeping sickness as *Glossina* spp. obtain transmit primary endosymbionts vertically from mother to progeny.

Another approach that is potentially applicable to the control of sleeping sickness would be to use *Wolbachia* spp., and the CI phenomenon that it can induce, to force the transgenic symbiont through the insect population. It has also been suggested that this method could be used to drive the transgenic symbiont into populations that are already infected with *Wolbachia* spp., by exploiting the CI that has been observed between different strains of the bacterium (Sinkins *et al.*, 1995; Beard *et al.*, 2001; Sinkins, 2004).

This approach also addresses ecological concerns as to the eradication of insect populations and the potential effects this action might have on the local ecosystem. Since the use of paratransgenic insects would not remove the insect from the environment there should be no detrimental effects of this programme on other wildlife. However, the implications of releasing genetically modified organisms into the environment need to be carefully considered, not only because of possible, unforeseen consequences that this might have but also because of the adverse public reaction it may cause.

1.7 List of objectives

1. To characterise the growth of *S. glossinidius in vitro* under various conditions such as altered incubation temperatures and iron-deficiency.

2. To examine the dynamics of siderophore production by *S. glossinidius* under iron-deficient and normal conditions.

3. To quantify the *S. glossinidius* population size and density *in vivo* and to examine the copy number of the extrachromosomal elements pSG1 to pSG4 in this bacterium.

4. To determine the prevalence of *S. glossinidius*, pSG1 to pSG4 and *Wolbachia* spp. in various species of wild tsetse and laboratory colonies.

5. To explore the possibility of horizontal transmission of *S. glossinidius*.

Chapter 2

Materials and methods

2 Materials and Methods

2.1 Materials

Materials were purchased from: Alpha Laboratories Limited (40 Parham Drive, Eastleigh, Hampshire, SO50 4NU, United Kingdom); Oxoid Ltd (Wade Road, Basingstoke, Hampshire, RG24 8PW, United Kingdom); Scientific Laboratory Supplies Limited (SLS) (Nottingham South and Wilford Industrial Estate, Nottingham, NG11 7EP, Nottinghamshire, United Kingdom).

2.2 Animals

Sheep were obtained from Easter Bush Farm (Easter Bush, Roslin, EH25 9RG, United Kingdom).

2.3 Chemicals

Chemicals were purchased from: Sigma Chemicals Ltd. (Fancy Road, Poole, Dorset, BH12 4QH, United Kingdom); Gibco BRL Ltd. (3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, United Kingdom); Invitrogen (Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, United Kingdom) Ambion (2130 Woodward Street, Austin, Texas, 78744-1832, USA).

2.4 Kits

DNeasy tissue kit, DNA and RNA extraction kit, RNeasy tissue kit, Qiagen shredder kit and QIAquick gel extraction kit were purchased from Qiagen (Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, United Kingdom).

2.5 Oligonucleotides and sequencing

Oligonucleotides for use in polymerase chain reaction were synthesised by Transgenomic Bioconsumables (Glasgow, UK).

Sequencing was carried out by GATC Biotech (Jakob-Stadler-Platz 7, Konstanz, 78467, Germany).

2.6 Sterilisation

Solutions and equipment unless otherwise stated were sterilised by autoclaving at 15 lb/in^2 at 121°C for 20 minutes. Trays used for tsetse feeding were sterilised by heating at 120°C for 4 hours.

2.7 Media composition

2.7.1 Luria Broth medium

10 g/l tryptone (Sigma, UK), 5 g/l yeast extract (Sigma, UK), 5 g/l NaCl (Sigma, UK).

2.7.2 Luria broth plates with ampicillin/IPTG/X-Gal

Luria broth (LB) medium with the addition of 15 g/l agar (Sigma, UK) sterilised by autoclaving before addition of 100 μg/ml ampicillin (Sigma, UK), 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (Sigma, UK) both filter sterilised and 80 μg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma, UK)

2.7.3 Mitsuhashi and Maramorosch basal medium

Mitsuhashi and Maramorosch (MMI) basal medium was made to the recipe of Mitsuhashi and Maramorosch (Mitsuhashi & Maramorosch, 1964).

MMI basal medium contains: 8 .125 g/l lactalbumin hydrolysate (Sigma, UK); 0.25 g/l $CaCl.2H_2O$ (Sigma, UK); 0.25 g/l KCl (Sigma, UK); 0.125 g/l $MgCl_2.6H_2O$ (Sigma, UK); 8.75 g/l NaCl (Sigma, UK); 0.15 g/l $NaHCO_3$ (Sigma, UK); 0.28 g/l $NaH_2PO_4.2H_2O$ (Sigma, UK) and 5 g/l D-glucose (Sigma, UK); 6.25 g/l yeastolate (Invitrogen, UK).

MMI was prepared without yeastolate or heat-inactivated foetal calf serum (FCS) (Invitrogen, UK) and then autoclaved to sterilise. Once cool, yeastolate and FCS to 20% (vol/vol) were added and the whole medium was filter sterilised.

MMI agar was prepared by adding 10 g/l low melting point agar (Sigma-Aldrich, UK) to MMI before the addition of yeastolate and FCS. After autoclaving the medium was cooled to 50°C in a water bath. Packed horse blood (Oxoid, UK), yeastolate and FCS were then filter sterilised and added prior to pouring the plates in a laminar flow cabinet.

2.7.4 Chrome azurol S solution

In order to make chrome azurol S (CAS) solution, 60.5 mg of CAS was dissolved in 50 ml deionised water. To this 10 ml of 1 mM $FeCl_3.6H_2O$ in 10 mM HCl and 72.9 mg of cetrimide dissolved in 40 ml deionised water was added. The solution was sterilised by autoclaving. In order to make CAS agar, 1 g low melting point agar (Sigma-Aldrich, UK) and 20 ml autoclaved CAS solution was added to 80 ml MMI and autoclaved.

2.8 Collection of blood for tsetse feeding

Six to seven year old ewes (Easter Bush Farm, UK) were rendered instantly insensible by captive bolt to the skull and bled by puncture of the jugular vein. Blood was then passed into a defibrinating unit and allowed to defibrinate for 15 minutes before being separated into 100 ml aliquots and stored at 4°C for 5 days.

2.9 Tsetse fly origin and maintenance of flies

2.9.1 Tsetse fly origin

Glossina morsitans morsitans (Westwood) were originally from the Langford colony established at Bristol from pupae collected from Zimbabwe in 1967.

Glossina palpalis palpalis (Robineau-Desvoidy), *Glossina fuscipes fuscipes* (Newstead), *Glossina brevipalpis* (Newstead) and *Glossina austeni* (Newstead) were kindly provided as pupae from cultures held at the Seibersdorf Laboratories, International Atomic Energy Agency, Austria and reared until eclosion.

2.9.2 Emergence of tsetse flies

Tsetse flies were kept at 25°C ± 1°C, at 70% relative humidity. Pupae were collected from deposition trays and placed into emergence cages. Once tsetse flies had emerged they were chilled at 4°C for 10 minutes and separated into groups of males and females. These flies were placed in cages and allowed to rest for 24 hours before being allowed to feed. Males were kept for 7-10 days before being used for mating, whilst females were mated immediately after their first blood meal. Flies were allowed to mate for 24 hours unless otherwise stated, then chilled, separated into groups of males and females once more and allowed to rest for 24 hours before feeding. Females were then kept on trays from which any deposited pupae were collected.

2.9.3 Feeding of tsetse flies

Tsetse flies were fed through an artificial silicon membrane system (Mews, 1980), three times a week. Defibrinated blood was poured onto trays on heating blocks (37˚C) and covered with a silicon membrane. Caged tsetse flies were then placed onto the membrane and the cages covered with a dark cloth. Feeding was allowed to continue for 10 minutes.

2.9.4 Wild flies

The wild flies used in this study were collected from two areas and at two time points one year apart, January 2004 and 2005 (see Figure 2.2 and 2.2).

Figure 2.1: Map of Tanzania showing Bushiri, where wild flies were collected.

Figure 2.2: Maps of Zimbabwe showing Rekomitjie Research Station, where wild flies were collected.

The first group of wild flies were collected from Tanzania and Zimbabwe in January 2004. Tanzanian flies were caught from Bushiri, near Pangani. This is approximately 50 km south of Tanga, a commercial and industrial port on the Indian Ocean. Zimbabwean flies were caught from Rekomitjie Research Station in the Zambezi valley, near Chiuyi River.

Four species of tsetse fly were screened: *G. austeni*; *G. brevipalpis*; *G.m.morsitans* and *G. pallidipes* (see Table 2.1). Flies were preserved in acetone for transport from

Africa to the UK. DNA was extracted by Gurdeep Lall, using the cetyltrimethylammonium bromide (CTAB) extraction protocol (see 2.9.4.1).

Species of *Glossina*	Country of origin	Sample number (male/female)
G. austeni (Newstead)	Tanzania	53 (19 / 34)
G. brevipalpis (Newstead)	Tanzania	17 (6 / 11)
G.m.morsitans	Zimbabwe	72 (31 / 41)
G. pallidipes (Austen)	Tanzania	88 (14 / 74)
G. pallidipes (Austen)	Zimbabwe	93 (32 / 61)

Table 2.1: Field-collected samples of *Glossina* spp. from Tanzania and Zimbabwe at the first time point.

2.9.4.1 DNA extraction protocol for field-caught flies (Lall, 2005)

A CTAB extraction protocol was used to extract DNA from these flies. Single individual flies were homogenised in 200 µl pre-warmed CTAB buffer at 60°C [5% CTAB, 1M Tris-HCl (pH8), 0.5M ethylenediaminetetraacetic acid (EDTA) (pH 8), 5 M NaCl, 18% distilled water, 20 µl β-mercaptoethanol]. The mixture was incubated at 60°C for 30 minutes. One volume of 24/1 chloroform/isoamyl alcohol was added and this was followed by gentle mixing for 5 minutes after which centrifugation at 8,000 rpm was carried out for 10 minutes. Next, one volume of isopropanol was added and the mixture was gently mixed and incubated at -20°C overnight. The tubes were centrifuged at 14,000 rpm for 10 minutes at 4°C. Pellets were washed with 1 ml of 70% ethanol and again centrifugation at 14,000 rpm was done at 4°C for 10 minutes. Depending on the size of DNA pellets obtained, they were dissolved in 20-50 µl distilled water.

Following extraction, the genomic DNA was quantified using a spectrophotometer (Model: Beckman Coulter DU 530) and working concentrations of 500 ng/µl were prepared then stored at -20°C. These samples were further diluted by 1 in 10 to give enough material to analyse.

The second group of wild flies was collected in January 2005 from Rekomitjie Research Station in Zimbabwe as before (see Table 2.2). The DNA was extracted from these flies using the DNeasy™ Kit (Qiagen, UK) by Alistair Darby.

Species of *Glossina*	Country of origin	Sample number (male/female)
G.morsitans	Zimbabwe	20 (8 / 12)
G. pallidipes (Austen)	Zimbabwe	25 (0 / 25)

Table 2.2: Field-collected samples of *Glossina* spp. from Zimbabwe at the second time point.

The samples were screened for *SODgroEL*, *P1 056*, *P2 26*, *P3 10* and *P4 01*, to give the prevalence of *S. glossinidius* in wild fly populations and the occurrence of each plasmid within *S. glossinidius* in the wild and each PCR was repeated in triplicate. The presence of *Wolbachia* spp. was also investigated using primers for the *Wolbachia* spp. surface protein, *wsp*.

2.9.5 Investigation of symbiont dynamics through the life cycle of *G. m. morsitans*

Pupae that had been deposited over a period of 48 hours were collected from the laboratory colony and placed in a puparium at 25°C ± 1°C, at 70% relative humidity. Six pupae were taken for DNA and RNA extraction at the time of collection and six more at seven day intervals. Upon eclosion, flies were chilled at 4°C for 10 minutes and separated into groups of males and females. From the population of adult flies,

three females and three males were sampled at seven day intervals (see Figure 2.3). Adult flies were fed as before (see 2.9.3) and were kept entirely separate from all other laboratory colony flies.

On collection, pupae and flies were surface sterilised in 1 ml of 5% bleach and subsequently washed twice in 1 ml of distilled water. Samples were homogenised in accordance with the manufacturers protocol for the Qiagen DNA/RNA kit (Qiagen, Crawley, UK) and then passed through a QIAshredder™ (Qiagen, Crawley, UK) in order to maximise tissue and cell disruption before continuing with the DNA/RNA kit protocol (see 2.15.3).

2.9.6 Collection of *G. brevipalpis* and *G. m. morsitans* pupae

Pupae that had been deposited over a period of 6 hours were collected from the *G. brevipalpis* and *G. m. morsitans* laboratory colonies. Ten *G. brevipalpis* pupae and nine *G. m. morsitans* pupae were immediately frozen at -80°C. The remaining pupae (nine individuals from each of the two *Glossina* species) were incubated at 25°C ± 1°C, at 70% relative humidity for seven days and then frozen at -80°C. DNA was extracted from all pupae as described in 2.15.1.

2.9.7 Collection of teneral flies

In order to further investigate the time point at which tsetse flies are most susceptible to infection with trypanosomes, larger samples of teneral *G. brevipalpis* and *G. m. morsitans* were taken for quantification of *S. glossinidius* populations by quantitative PCR (qPCR) (see Figure 2.3). A group of fed flies was sampled at ten days post-eclosion for comparison.

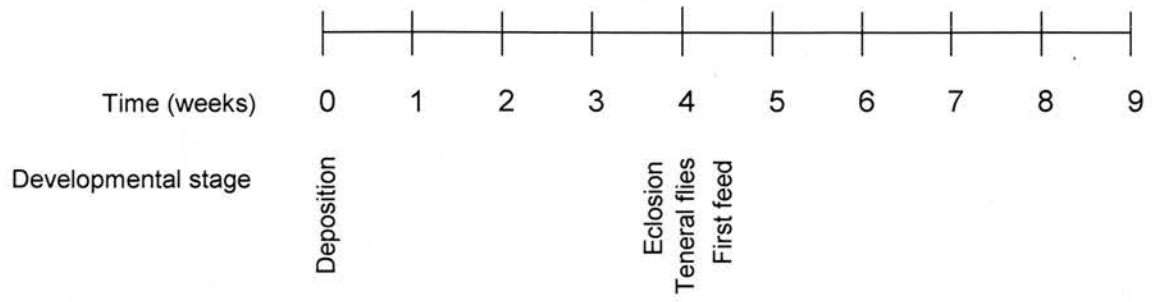


Figure 2.3: Data points and developmental stages for the collection of samples throughout the lifecycle of the tsetse fly.

Flies that had emerged over a period of four hours were chilled at 4˚C for 10 minutes and separated. Twenty-seven *G. m. morsitans* and twenty-four *G. brevipalpis* males were taken and rested for one day at 25˚C ± 1˚C, at 70% relative humidity. The remaining twenty-five *G. m. morsitans* and twenty-four *G. brevipalpis* were kept for ten days as described in 2.9.2 and 2.9.3. All flies were weighed and then surface sterilised in 1 ml of 5% bleach and subsequently washed twice in 1 ml of distilled water. DNA was extracted using the Qiagen DNeasy kit (Qiagen, Crawley, UK) (see 2.15.1).

2.9.8 Collection of *G. austeni* and *G. p. palpalis* for quantitative PCR

Five each of *G. austeni* and *G. p. palpalis* teneral males were collected upon emergence and allowed to rest for one day at 25˚C ± 1˚C, at 70% relative humidity. All flies were weighed and then surface sterilised in 1 ml of 5% bleach and subsequently washed twice in 1 ml of distilled water. DNA was extracted using the Qiagen DNeasy kit (Qiagen, Crawley, UK) (see 2.15.1).

2.10 Isolation and cultivation of *Sodalis glossinidius* from *Glossina* spp.

2.10.1 Bacterial culture and strains

Bacteria were isolated from *Glossina* spp. haemolymph by the removal of the fly's head and collection of c. 2 µl of haemolymph with a microcapillary tube. The haemolymph was then inoculated into a sterile 1.5 ml eppendorf containing 1 ml of liquid MMI medium (2.7.3). This was incubated without shaking at 26.5°C for 24 hours prior to plating 250 µl of culture onto 10% packed horse blood MMI agar. Once isolated, strains of *S. glossinidius* were routinely subcultured onto MMI agar supplemented with 10% blood.

Isolation of *S. glossinidius* from *G. brevipalpis* was unsuccessful following the above protocol and so haemolymph from multiple individuals was inoculated into 1 ml MMI medium in order to increase the likelihood of inoculation with a viable bacterium. The protocol for isolation was continued as before from this point on. Morphological differences in the size of individual colonies on MM agar prompted the random selection of 100 individual colonies for further study.

All solid culture was carried out in a microaerobic atmosphere generated using the Campygen pack system (Oxoid, UK) which provided 5% O_2, balanced with CO_2 at 26.5%. Individual colonies from each clone were taken from these plates and grown in tightly sealed cell culture flasks (closed cap, 25 cm^2, Nunclon^{TM} Δ (SLS, UK)) containing 10 ml MMI liquid medium at 26.5°C laid horizontally without shaking. All subsequent liquid culture was carried out according to this method.

Stock strains were grown to an OD of c. 0.4 (measured at a wavelength of 600 nm) and 5 ml of each strain was taken to make a glycerol stock. A 1:1 ratio of 30% glycerol culture to bacterial culture was used and stored at -80°C.

For the purpose of these experiments, strains of *S. glossinidius* were given a nomenclature to distinguish between those isolated from different species of *Glossina* as shown in Table 2.3.

Strain Name	**Species of *Glossina***
GM	*G. m. morsitans*
GA	*G. austeni*
GP	*G. p. palpalis*
GF	*G. f. fuscipes*
GB	*G. brevipalpis*

Table 2.3: Strain nomenclature for isolates of *Sodalis glossinidius* from different species of *Glossina*.

2.10.2 Isolation of *S. glossinidius* from *G. m. morsitans* excretions

Fifteen male *G. m. morsitans* flies were separated and surface sterilised in 5% bleach. Flies were fed the next day on sterile ovine blood. After feeding flies were placed into ventilated bijoux and urine was collected after 1 hour. A loop of urine was inoculated onto MMI blood agar and incubated at 26.5°C microaerophilically. As a control a loop of the blood that was fed to the flies was also inoculated onto blood agar and incubated at 26.5°C microaerophilically.

Faeces were collected from the individual flies 48 hours after feeding. This was solubilised in 20 µl sterile saline and inoculated onto MMI blood agar as before.

Plates were examined for bacterial growth after 7 days. Individual colonies were suspended in 10 µl sterile water and boiled at 100°C for 10 minutes. The resulting sample was used as a template for PCR screening with *SOD16S* primers (see 2.17.1) to confirm that the bacterial growth was *S. glossinidius*.

2.11 Enumeration of bacteria

2.11.1 Bacterial viable cell counts

Ten ml MMI media supplemented with 20% foetal calf serum was inoculated with a single colony of *S. glossinidius* taken from a stock plate not more than a week old. Cultures were incubated at 26°C and samples were taken daily. Measurements of optical density (OD) (600 nm) were performed on a Beckman Coulter DU530 spectrophotometer and serial dilutions were made in phosphate buffer solution (PBS) pH 7.5. The number of viable cells per ml was established using the Miles Misra technique (Miles & Misra, 1938).

2.11.2 Total bacterial cell enumeration

Samples were serially diluted in PBS pH 7.5. Total cell counts of bacterial cultures were made using an haemocytometer.

2.11.3 Enumeration of copies of *SODgroEL* per *S. glossinidius* cell

Cultures of *S. glossinidius* GA were grown for 5 days until the culture reached an optical density of approximately 0.3 at 600 nm. In order to establish the number of copies of *SODgroEL* present in a single *S. glossinidius* cell, aliquots were taken from this bacterial culture to perform total cell counts and DNA extraction for qPCR. The number of copies of *SODgroEL* in the culture was measured using qPCR (see 2.19).

2.12 Bacterial growth curves

2.12.1 Growth of *S. glossinidius* isolates GA, GF, GM and GP.

Single colonies of each *S. glossinidius* strain were picked from stock plates and inoculated into liquid MMI (20% FCS). Cultures were grown to an OD of approximately 0.4 and 100 µl of the culture was used as inoculum. Each strain was grown in triplicate in 10 ml liquid MMI (20% FCS) at 26.5°C and 100 µl samples were aseptically removed daily and their OD measurements taken as before (2.11.1). Uninoculated liquid MMI (20% FCS) was used as a negative control.

2.12.2 Growth of 100 *S. glossinidius* GB isolates

Single colonies of *S. glossinidius* GB were picked from stock plates and inoculated into liquid MMI (20% FCS). Each of the 100 isolates originally collected were grown in 10 ml liquid MMI (20% FCS) at 26.5°C for four days. At this time the OD of each culture was measured as before. *S. glossinidius* GA was included as a positive control and uninoculated liquid MMI (20% FCS) was used as a negative control. Due to the large number of isolates no individual replicates were performed.

2.12.3 Growth of ten *S. glossinidius* GB isolates

Ten of the 100 *S. glossinidius* GB isolates were randomly selected and single colonies from stock plates inoculated into liquid MMI (20% FCS). Each isolate was grown in triplicate as described in 2.11.1. The siderophore production of these ten isolates was measured alongside the measurement of growth using the protocol described in 2.14.2.

2.12.4 Growth of *S. glossinidius* at three different incubation temperatures

S. glossinidius GA was grown as described in 2.12.1. Cultures were separated into three groups, each of which was incubated at a different temperature (20°C, 26.5°C or 30°C). Three replicates were included for each temperature as well as an uninoculated negative control. Optical density measurements were made as described in 2.11.1.

2.13 Iron-limitation and supplementation of growth media

2.13.1 Measurement of siderophore production by different isolates of *S. glossinidius*

S. glossinidius isolates were grown as described in 2.12.1 in MMI subjected to different treatments. In the first group iron was removed from the medium by treatment with chelex resin which was subsequently filtered off. The second group the medium was untreated and in the third it was supplemented with 50 µl $FeCl_3.6H_2O$. Equal volumes of culture supernatant and CAS solution were incubated at room temperature for 1 hour. Measurements of optical density (630 nm) were performed on a Bio Whittaker EL_X808. Each treatment had an uninoculated negative control and was replicated three times.

2.13.2 Growth of *S. glossinidius* under iron-depleted conditions

2.13.2.1 Inhibition of *S. glossinidius* growth

S. glossinidius GP were grown as described in 2.12.1 in MMI subjected to different treatments. In the first group the growth medium was depleted of iron by the addition of 10 mM nitrilotriacetic acid trisodium salt (NTA), a strong synthetic chelator. In the second group, the growth medium was depleted of iron as before and

then supplemented with 0.1 M of each of $MnCl_2$, $MgCl_2$, $CaCl_2$ and $ZnCl_2$ (Eichenbaum *et al.*, 1996). In the third group the medium was left untreated and this group acted as a positive control. Uninoculated medium was used as a negative control for each group. Measurements of OD were taken as described in 2.11.1.

2.13.2.2 Restoration of *S. glossinidius* growth

S. glossinidius was grown in MMI supplemented with 20% calf foetal serum for three days, until early exponential phase was reached. On day three the samples were split into three groups; one was untreated and acted as a positive control, another was treated with 10 mM NTA and the last was treated with both 10 mM NTA and 5 mM $FeCl_3.6H_2O$. Measurements of optical density (600 nm) were performed as before (2.11.1). Each treatment had an uninoculated negative control and was replicated three times.

2.14 Chrome azurol S assays for siderophore production

2.14.1 Siderophore indicator agar

S. glossinidius GA, GM and GP were inoculated onto CAS agar plates. Heat-killed *S. glossinidius* GM was used as a negative control. Plates were incubated at 26.5°C as described in 2.12.1. In the presence of siderophores the agar will change from blue to orange, giving a qualitative measurement of siderophore production. After four days the colour change of the CAS agar was photographed.

2.14.2 Liquid CAS assay for siderophore production

One mL of culture was extracted and centrifuged at 10000 rpm for 10 minutes at room temperature. From this 500 µl of supernatant was carefully taken off and

mixed with 500 µl of CAS solution. This mixture was incubated at room temperature for 1 hour and the OD of the sample measured at 630 nm in a Bio Whittaker EL_X808. Negative controls of uninoculated media were set up alongside and used to determine the % siderophore units in the sample by use of the calculation:

((reference – sample) x 100) / reference

(Schwyn & Neilands, 1987)

Where 'reference' is the OD of the uninoculated control and 'sample' is the OD of the bacterial culture sample.

2.14.3 CAS standard curve

A standard curve of CAS colour change in liquid medium was created using serial dilutions of $FeCl_3.6H_2O$ and EDTA in MMI medium to give 50 mM, 10mM, 5mM, 1mM, 0.5mM, 0.1mM and 0.05mM solutions. The optical density of the sample was measured as described in 2.14.2. 'Siderophore' units were calculated as in 2.14.2 using MMI medium as a reference.

2.15 Nucleic acid extraction

2.15.1 DNA extraction

The DNeasy kit (Qiagen, Crawley, UK) was used and the manufacturer's protocol followed for "Gram negative bacteria" and "insect" respectively, to extract DNA from cultures of *S. glossinidius* and from *Glossina* spp. DNA from *S. glossinidius* was extracted from 1 ml bacterial culture on day 3 of incubation. DNA from *Glossina* spp. was extracted from both pupae and adult flies by initially homogenising the insect for 30 seconds. DNA was used as a template for the amplification of gene fragments.

2.15.2 RNA extraction

The RNeasy kit (Qiagen, Crawley, UK) was used and the manufacturer's protocol followed, to extract RNA from cultures of *S. glossinidius* and from *Glossina* spp.

2.15.3 Simultaneous DNA and RNA extraction

The DNA / RNA kit (Qiagen, Crawley, UK) was used and the manufacturer's protocol followed, to extract DNA and RNA simultaneously from *Glossina* spp.

2.15.4 Boil preparations for bacterial DNA

To extract DNA from bacterial colonies grown on solid media for screening, individual colonies were taken and suspended in 10 µl of autoclaved distilled H_2O. This was boiled at 100°C for 10 minutes and stored at -20°C.

2.16 DNA quantification

DNA was quantified using PicoGreen (Molecular Probes, UK) following the manufacturer's protocol. Both high and low concentration standard curves were prepared using lambda DNA supplied with the kit. Dilutions of sample DNA were prepared in nuclease-free Tris-EDTA buffer to a dilution of 1 in 100 parts and 500 µl was mixed with 500 µl of the PicoGreen reagent mixture. Fluorescence was measured in a Perkin Elmer Luminescence Spectrometer LS50B.

2.17 Polymerase chain reaction

2.17.1 Primers

Gene name	Primer code	Primer sequence	Product size	Annealing temp. (°C)	Accession no.
S. glossinidius groEL (*mopB*)	SODgroELf SODgroELr	5' -CCA AAG CTA TCG CTC AGG TAG G 5' -TTC TTT GCC CAC TTT CGC CAT A	95bp	58	AF326971
S. glossinidius 16S	SOD16Sf SOD16Sr	5' –ACG GTC CAG ACT CCT ACG 5' –GCT AAC GCC TTC TTC CC	133 bp	55	AP008232
Glossina spp. α-elongation factor	TseAf TseAr	5' –CGG CTG GCA CGG TGA TAA CAT 5' –GCG GGA GGG TGG CAA CAT T	140 bp	57	A. Darby, pers.comm.
S. glossinidius pSG1	P1-103f P1-103r	5' –GAA TGC GAG GAT GAC A 5' –TTT TCA CGG ATA CG	110bp	52	AP008233
	P1-020xf P1-020xr	5' –ACA TAA TAG AGG GAT TCA G 5' –TGT CGT GTT GGT AAG GTG	189bp	52	AP008233
	P1-056f P1-056r	5' –ATT TGT GAG GTG TCT GAG G 5' –GAA GAT GCT ACG GAG GAT A	139bp	52	AP008233
S. glossinidius pSG2	P2-26f P2-26r	5' –AGT TGT AGC ACA GCG TGT A 5' –TGA AGT TGG GAA TGT CG	120bp	52	AP008234
	P2-13-vef P2-13-ver	5' –ACC CAG CAC AAG ACA TTA 5' –CCC GAG CCA ACA TAC AC	142 bp	52	AP008234
S. glossinidius pSG3	P3-10f P3-10r	5' –AGG ACG AGC CAT ACG C 5' –CTG GGA CAG TGG TAA CGA TG	227bp	56	AP008235
	P3-10/11f P3-10/11r	5' –CCA TAT CAG GCA CCC AG 5' –CAG TCC ACC ACC CGT AA	183 bp	52	AP008235
S. glossinidius pSG4	P4-01f P4-01r	5' –GGCGTT TAT TAT GAG GGT 5' –GAT GGT ATT TAT CTT TCC GTA G	195bp	52	AS868439
	P4-05f P4-05r	5' –TCC TGT CGT GCT CCT CG 5' –TGC CGC TTG AAT GGG T	159 bp	52	AS868439
	P4-09f P4-09r	5' –CTC TTC ACT CGG AGG GTT 5' –TTC TGA GCC AGG GAT GT	136bp	52	AS868439
Wolbachia spp.	wsp 81F wsp 691R	5'-TGGTCCAATAAGTGATGAAGAAAC 5'-AAAAATTAAACGCTACTCCA	610 bp	54	AF020079

Table 2.4: Primers used for PCR analysis. Primers for *Glossina* spp. α-elongation factor and *S. glossinidius* pSG1 – 4 were designed by Dr. A. Darby.

2.17.2 Specificity of *SodGroEL* primers

The specificity of the *S. glossinidius* GroEL primers was confirmed by sequencing PCR products derived from tsetse DNA and testing the primers for mis-priming against the related bacterial species *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

2.17.3 PCR reagents

A standard PCR reagent mixture was used for all PCR, unless otherwise stated, which consisted of 1x PCR buffer (Invitrogen, UK), 2 mM $MgCl_2$ (Invitrogen, UK), 0.2 mM dNTPs (Promega, UK), 20 µM each primer (Qiagen, UK) and 1 U Platinum® *Taq* polymerase (Invitrogen, UK).

2.17.4 PCR parameters

Reaction conditions were 30 cycles of denaturation at 94°C for 1 minute; annealing at the appropriate temperature for the primer (see Table 2.4) for 1 minute; and elongation at 72°C for 1 minute. In addition there was an initial denaturation step of 94°C for 3 minutes and a final elongation at 72°C for 5 minutes.

2.17.5 Gel preparation

Ten µl of sample was loaded into a 2% tris borate EDTA gel and run at 100 V for 45 – 60 minutes. Gels were stained in ethidium bromide for 15 minutes and destained in water for 15 minutes. They were then visualised under ultraviolet light using the Biorad Gel Doc 2000.

2.17.6 Sequencing

PCR products were checked by gel electrophoresis (see 2.17.5) and bands of interest were excised from the gel under ultraviolet light using sterile scalpels. The PCR product was purified from the gel using a QIAquick gel extraction kit (Qiagen, UK)

following the manufacturer's protocol. Purified product was sent to GATC Biotech (Konstanz, Germany).

2.18 Screening of tsetse flies for trypanosome infections

2.18.1 Infection of flies

2.18.1.1 Trypanosome stocks used in the current work

T. b. brucei isolate Buteba 135 (BUT 135) was isolated from a cow in Buteba village, Uganda in 1990 (Dale *et al.*, 1995)

2.18.1.2 Infection of tsetse flies from bloodstream stabilates

Twenty-five male *G. m. morsitans* were collected upon emergence, had their wings clipped and were allowed to rest for 24 h before being infected with trypanosomes. To make up the infective blood meal from a stabilate, it was defrosted at room temperature then reconstituted in 5 ml of ovine blood. The blood meal was then placed on a heated tray and covered with a silicon membrane. Tsetse were then placed on the membrane and allowed to feed. Flies that did not feed were removed from the experiment.

2.18.2 Microscopic diagnosis of trypanosome infection

Flies were chilled at 4°C for 30 minutes and then kept on ice until dissection. Flies were dissected in saline 10 days after infection and their midguts examined for the presence of trypanosomes by phase-contrast microscopy (x400).

2.18.3 PCR diagnosis of trypanosome infection

2.18.3.1 PCR reagents

PCR for *Trypanosoma brucei* were carried out in standard reaction volumes of 25 µl containing the following reagents: 16.0 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL (pH 8.8 at 25°C), 0.01% Tween 20 (NH_4) buffer (Bioline, London, UK), 1.5 mM Mg^{2+}, 200 µM deoxynucleotide triphosphates, 0.7 Units BIO*TAQ* RED™ DNA polymerase (Bioline, London, UK) and 0.4 µM of each primer (Table 2.5).

Primer code	Primer sequence	Product size	Annealing temperature (°C)	Reference
TBR1 TBR2	5'-CGA ATG AAT ATT AAA CAA TGC GCA GT 5'-AGA ACC ATT TAT TAG CTT TGT TGC	173bp	55	Moser *et al.*, 1989

Table 2.5: Primers used for PCR diagnosis of trypanosome infection.

2.18.3.2 PCR parameters

Reaction conditions were 30 cycles of denaturation at 94°C for 60 seconds; annealing at 55°C for 60 seconds; and elongation at 72°C for 30 seconds. In addition there was an initial denaturation step of 94°C for 3 minutes and a final elongation at 72°C for 5 minutes.

2.19 Quantitative PCR

Quantitative PCR is an amplification that uses fluorescent dyes and optical technology to measure the production of DNA throughout the PCR. One such dye is SYBR Green I (Qiagen, Crawley, UK), a compound that yields a strong fluorescent

signal upon binding double-stranded DNA. This signal is measured after each cycle of PCR amplification and allows the quantification of DNA in the sample by comparison to DNA standards.

2.19.1 Production of quantification standards

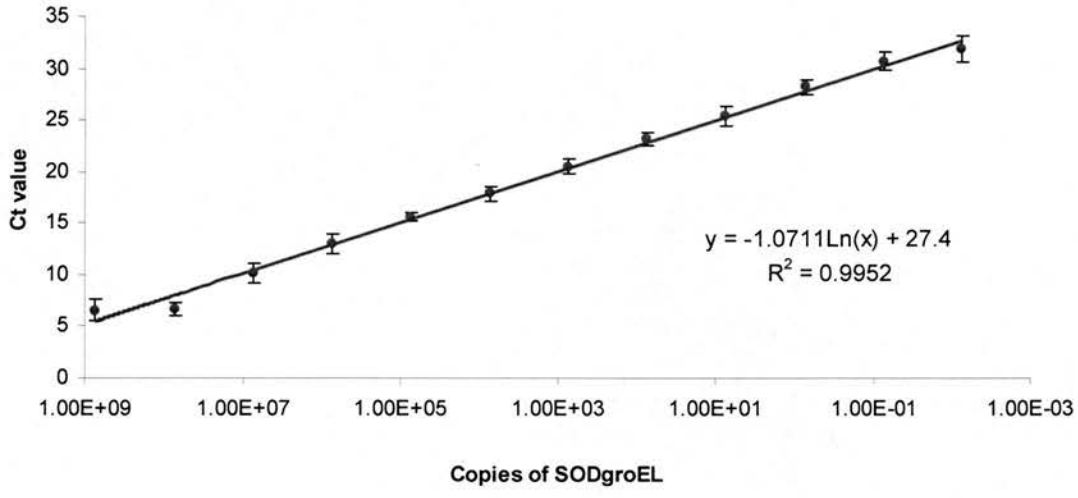
2.19.1.1 PCR product quantification standards

Quantification standards for qPCR were initially made from PCR amplification product of the relevant gene. PCR amplification products for *SODgroEL*, *P1 103*, *P2 26*, *P3 10* and *P4 09* were run out on 2% agarose gels and bands were cut out and purified using the QIAquick gel extraction kit (Qiagen, UK). Amplification product was sent for sequencing by GATC Biotech (Konstanz, Germany). The DNA concentration of the product was measured as described in 2.16 and the number of copies of the target sequence present were calculated. Serial dilutions of amplification product were made in nuclease-free distilled H_2O. These standards were used in the quantification of the copy number of extrachromosomal elements in *S. glossinidius* both *in vivo* and *in vitro.*

2.19.1.2 Plasmid quantification standards

In later experiments quantification standards were made from cloned plasmids (see Graph 2.1). PCR amplification products were run out on 2% agarose gels and bands were cut out and purified using the QIAquick gel extraction kit (Qiagen, UK). Amplification product was sent for sequencing by GATC Biotech (Konstanz, Germany). Upon confirmation of the sequence the products were cloned into plasmid vectors using the pCR2.1 TOPO cloning kit (Invitrogen, UK). Cloning was confirmed by digestion with *EcoR1*. Cultures of the transformed *E. coli* were grown up and plasmid DNA was extracted by miniprep and aliquoted (Qiagen, UK). This DNA was quantified using PicoGreen (see 2.16) and the number of copies of the

target sequence of interest calculated. These standards were used in all qPCR experiments except for those stated above in 2.19.1.1.



Graph 2.1: Standard curve for qPCR *SodGroEL* quantification standards.

2.19.2 Primers

Gene name	Primer code	Primer sequence	Product size	Annealing temp. (°C)	Accession no.
S. glossinidius groEL (mopB)	SODgroELf	5' -CCA AAG CTA TCG CTC AGG TAG G	95bp	58	AF326971
	SODgroELr	5' -TTC TTT GCC CAC TTT CGC CAT A			
Glossina spp. α-elongation factor	TseAf	5' –CGG CTG GCA CGG TGA TAA CAT	140 bp	57	A. Darby, pers.comm.
	TseAr	5' –GCG GGA GGG TGG CAA CAT T			
S. glossinidius pSG1	P1-103f	5' –GAA TGC GAG GAT GAC A	110 bp	52	AP008233
	P1-103r	5' –TTT TCA CGG ATA CG			
	P1-056f	5' –ATT TGT GAG GTG TCT GAG G	139 bp	52	AP008233
	P1-056r	5' –GAA GAT GCT ACG GAG GAT A			
S. glossinidius pSG2	P2-13-vef	5' –ACC CAG CAC AAG ACA TTA	142 bp	52	AP008234
	P2-13-vef	5' –CCC GAG CCA ACA TAC AC			
	P2-26f	5' –AGT TGT AGC ACA GCG TGT A	120 bp	52	AP008234
	P2-26f	5' –TGA AGT TGG GAA TGT CG			
S. glossinidius pSG3	P3-10	5' –AGG ACG AGC CAT ACG C	227 bp	56	AP008234
	P3-10	5' –CTG GGA CAG TGG TAA CGA TG			
S. glossinidius pSG4	P4-01	5' –GGC GTT TAT TAT GAG GGT	195 bp	52	AS868439
	P4-01	5' –GAT GGT ATT TAT CTT TCC GTA G			
	P4-05	5' –TCC TGA GCC AGG GAT GT	159 bp	52	AS868439
	P4-05	5' –TGC CGC TTG AAT GGG T			

Table 2.6: Primers used for quantitative PCR analysis. Primers for *Glossina* spp. α-elongation factor and *S. glossinidius* pSG1 – 4 were designed by Dr. A. Darby.

2.19.3 PCR parameters

qPCR was performed in an Opticon Monitor system (Bio-Rad, UK). Reaction volumes of 10 µl contained 5 µl of Qiagen QuantiTect SYBR Green PCR Master Mix, 1µl sterile water, 0.5 µl each of 10 µM forward and reverse primers, 2 µl Q-Solution and 1 µl of target DNA in single wells of a 96 well plate (Bio-Rad). The primers used for the selective amplification of a short length of the *S. glossinidius* GroEL gene (95 bp), the *Glossina* spp. α-elongation factor gene (121 bp) and pSG1 p1 103 (110 bp) are listed in Table 2.6. PCR parameters comprised an initial activation step of 95°C for 15 mins, followed by 50 cycles of 95°C for 45 seconds, primer annealing for 45 seconds and elongation at 72°C for 45 seconds. Annealing temperatures are listed in Table 2.6. At the end of the 50 cycles an elongation step at 72°C for 5 minutes was included. A melting curve analysis was performed at the end of the PCRs to check for non-specific amplification and primer dimers. qPCR was performed in triplicate on all samples for each primer set.

Data was presented as "copies per insect" to give a measurement of the actual numbers of gene copies in a single tsetse. DNA concentrations in corresponding samples were compared to ensure that there were no anomalies. Data for comparison of *G. m. morsitans* and *G. brevipalpis* was also normalised by the DNA concentration of the sample and presented as "copies per ng DNA". This was done in order to compare the copy numbers relative to the size of the fly as *G. brevipalpis* are far larger than *G. m. morsitans.*

2.20 Reverse transcription PCR assays

Reverse transcription PCR allows the detection of RNA in the sample. RNA is extracted from the sample using a RNeasy ™ kit (Qiagen, Crawley, UK) and treated with reverse transcriptase alongside a specific primer. This produces a single copy of cDNA of the desired RNA sequence for each copy present in the sample. The cDNA

is then amplified through PCR or qPCR. This allows the investigation of gene expression in bacteria.

Reverse transcription was performed using SuperScript™ III reverse transcriptase (Invitrogen, Paisley, UK) and DNA-free™ (Ambion, Texas, USA), DNase treatment and removal reagents. RNA was extracted from *S.glossinidius* GA, GM and GP cultures at three days post-inoculation (2.15.2) and treated with DNase (2.20.1). *P1 103* antisense primers (10 mM) were used during the reverse transcription step and the resulting cDNA was used as template for PCR amplification of *P1 103* as described in 2.17.

2.20.1 Treatment of RNA samples with DNase

Ambion® Turbo DNA-free™ was used to remove any DNA contamination from RNA samples before reverse transcription. To 100 µl RNA elutate, 10 µl DNase 1 buffer and 1µl DNase 1 (2 units) was added. The sample was gently mixed and incubated at 37°C for 20 – 30 minutes. The DNase Inactivation Reagent was resuspended by flicking the tube and 11.1 µl of the slurry was added to the sample. The sample was incubated for 2 minutes at room temperature and then centrifuged at 10,000 x g for 1 minute to pellet the DNase Inactivation Reagent..

2.21 Statistical analyses

2.21.1 Growth curves

Linear mixed effect models were carried out on log transformed data, with the sample the bacteria came from entered as a random effect, incubation time entered as a fixed covariate effect and isolate entered as a fixed effect. The data was transformed in order to normalise the residuals.

2.21.2 CAS assay

Student's t-tests were used to compare the mean siderophore production units between *S.glossinidius* grown under different conditions and to compare the change in mean siderophore units over time.

2.21.3 Quantitative PCR

Data were analysed using Minitab v. 14 for Windows. Mann-Whitney *U* tests were performed to determine whether target gene copy numbers significantly differed between time points, sexes and species. Index of dispersion tests were performed in order to assess whether the data were randomly dispersed or aggregated.

2.21.4 Repeatability of screening

The results obtained in this study for the screening of samples for various characteristics were tested for their 'repeatability' in order to determine the value of the diagnostic test. Since the screening was performed in triplicate for each sample, Cochran's Q test (Equation 2.1) was used in order to assess the agreement between the repeated results (Thrusfield, 1995). The sampling distribution of Q was approximated by the χ^2 distribution with k-1 degrees of freedom.

$$Q = \frac{(k-1)[k\sum G^2 - (\sum G)^2]}{k\sum L_i - \sum L_i^2}$$

Equation 2.1: Cochran's Q test – where k is the number of experimental runs; G is the total number of positive results in each experimental run and L_i is the total number of positive results for each individual sample.

Where the P value statistic was greater than 1% significance, the proportion of positive and negative results was seen to differ significantly between the repeated experimental runs and the repeatability of the diagnostic test was found to be low. In such cases the data was reanalysed using an absolute rule (3/3 rather than 2/3 for a positive result).

2.21.5 Fisher's exact test

To evaluate the difference in the prevalence of *S. glossinidius* and the four extrachromosomal elements in wild and laboratory flies, as well as in *S.glossinidius* GB isolates, Fisher's exact tests were employed.

2.21.6 Screening of wild flies for *S. glossinidius and Wolbachia* spp.

To investigate the difference in the prevalence of *S.glossinidius*, the extrachromosomal elements pSG1 – pSG4 and *Wolbachia* spp. in various populations of *Glossina* spp, screening data was analysed in R using generalised linear mixed effect models with binomial errors. This was because this study is interested in the presence or absence of these bacteria and extrachromosomal elements in relation to each other.

In order to take account of the effect of individual flies this was entered in as a random effect, whereas country, species, primer and sex were entered as fixed effects. The code used for analysis is shown in Box 2.1 and Box 2.2, and differences were considered significant at $p<0.05$.

```
anova(glm(Infected~Country+Species+Gender+Timepoint+Species:Timepoint,data=MollyWolbachia,family="binomial"),test="Chisq")
```

Box 2.1: Code used for the analysis of the prevalence of *Wolbachia* infection in field-collected *G. pallidipes* and *G.morsitans* from Zimbabwe and Tanzania (glm = general linearised model; infected = infection status of individual flies; country = Zimbabwe or Tanzania; gender = gender

of individual flies; timepoint = time point at which flies were collected; species = *G. pallidipes* or *G.morsitans*; data = the dataset analysed).

```
anova(glm(Infected~Species+Gender+Timepoint+Species:Timepoint,data=MollyWolbachia,family="binomial",subset=Country=="Zimbabwe"),test="Chisq")
```

Box 2.2: Code used for the analysis of the prevalence of *Wolbachia* infection in field-collected *G. pallidipes* and *G.morsitans* from Zimbabwe at two time points (glm = general linearised model; infected = infection status of individual flies; gender = gender of individual flies; timepoint = time point at which flies were collected; species = *G. pallidipes* or *G.morsitans*; data = the dataset analysed).

Chapter 3

Growth kinetics and siderophore production

3 Growth kinetics and siderophore production

3.1 Introduction[1]

3.1.1 Bacterial growth dynamics

Bacterial replication is reliant upon the ability of the bacterial cell to form new protoplasm from the nutrients that are available in the surrounding environment. It mainly takes place through the process of binary fission, thus creating clonal populations. In a closed system, or batch culture, bacterial growth can be divided into several phases: lag phase, exponential phase, and stationary phase (Figure 3.1). It has also recently been expanded by Finkel to include two additional phases: death phase and long-term stationary phase (Finkel, 2006). Death phase is a period characterised by the loss of viable cells after a prolonged period of stationary phase and occurs when the limiting growth factors in the medium, such as nutrients, are expended, or when waste metabolites build up to toxic levels. This phase may be delayed by the addition of fresh growth medium to the culture, a technique known as "batch culture". Long-term stationary phase is a period of indefinite duration during which 'birth' and death rates are balanced.

---

[1] The work presented in this chapter has formed the basis of two published papers Darby, A. C., Lagnel, J., Matthew, C. Z., Bourtzis, K., Maudlin, I. and Welburn, S. (2005). Extrachromosomal DNA of the symbiont *Sodalis glossinidius*. *Journal of Bacteriology* **187**(4): 5003 - 5007, Matthew, C. Z., Darby, A. C., Young, S. A., Hume, L. H. and Welburn, S. (2005). The rapid isolation and growth dynamics of the tsetse symbiont *Sodalis glossinidius*. *FEMS Microbiology Letters* **248**(1): 69 - 74..

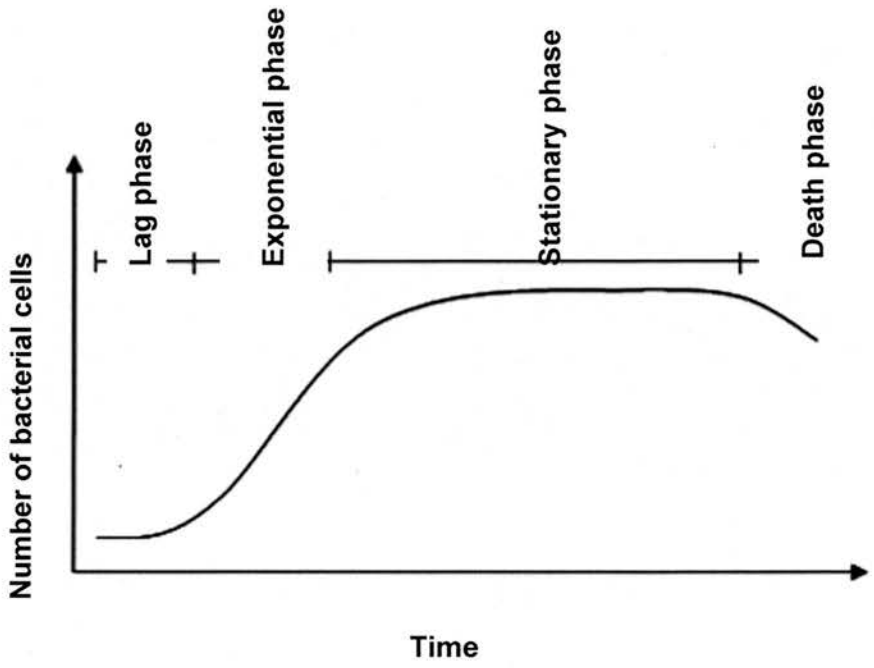


Figure 3.1: Typical bacterial growth curve showing the phases: lag, exponential (or log), stationary and death.

3.1.2 Factors affecting bacterial growth dynamics

During lag phase bacteria are adjusting to their new environment. The length of lag phase is dependent on many factors including the inoculum size, recovery from any shock inflicted during the inoculation and synthesis of new enzymes.

When the bacteria are able to start dividing regularly the culture enters exponential phase until the growth of the bacteria becomes limited, at which point stationary phase is reached. During exponential phase, the bacterial cells are dividing at a constant rate, the speed of which depends on the conditions of incubation, such as

temperature, and the composition of the growth medium. Bacterial growth in a batch culture will eventually become limited by the exhaustion of the nutrient supply, exhaustion of space or the accumulation of inhibitory metabolites or end products. Eventually stationary phase is followed by a gradual decrease of viable cells as the bacteria die and are not replaced.

3.1.3 Measurement of bacterial growth

The measurement of bacterial growth can be conducted in terms of either cell mass or cell numbers. The quantification of the turbidity, or OD, of a cell suspension is performed by using a spectrophotometer to measure the amount of light of a certain wavelength that is scattered by the cell suspension. Bacteria, along with other particulate objects, will scatter light in direct proportion to their numbers and after construction and calibration of a standard curve, bacterial numbers may be quantified. The sensitivity of this technique is limited to a maximum of approximately 10^7 cells per ml.

Bacterial cell numbers can be measured directly or indirectly. Total cell numbers, including both live and dead cells as they are indistinguishable, can be counted using a microscope and a counting chamber. In order to count only viable cells, indirect plate counts can be performed. In this technique, dilutions of a cell suspension or culture are plated on a suitable medium. Each viable unit grows to form a colony which can be counted and is referred to as a colony forming unit (CFU). This has the limitation that clumps or chains of cells will develop into only a single colony.

3.1.4 Bacterial iron metabolism

Iron is an essential nutrient for the growth of the majority of organisms, yet in the presence of oxygen it can have toxic effects by catalysing the generation of free radicals. These destructive oxidative reactions can destroy membranes and damage nucleic acids. Insects, in common with all other organisms, have developed mechanisms to deal with the negative effects of iron. Iron-binding proteins, such as transferrin and ferritin are used to sequester ferric ions and protect the insect against iron-overload respectively (Law, 2002). Transferrin has a role in the innate immune response of the insect to bacterial infection.

The link between iron and infection was identified around 60 years ago when Schade and Caroline found that iron could negate the bacteriostatic effect of ovotransferrin (Schade & Caroline, 1944). Iron is often sequestered by iron-binding proteins in the host and very little, approximately only 10^{-18} M ferric iron is freely available, limiting the amount for bacterial growth (Andrews *et al.*, 2003). In order to survive in iron-depleted environments, bacteria have developed an iron-uptake system that is characterised by the production of ferric iron high-affinity ligands and the specific transport of the ferric complex. These ligands, called siderophores, are low molecular weight compounds that range from 400-1000 Dalton.

The addition of chelating agents to culture medium makes iron inaccessible and can reduce the growth rate of bacteria that do not produce siderophores to almost nothing (Expert, 1999). Many pathogenic bacteria, such as *Pseudomonas* spp. and uropathogenic *E. coli* use siderophores to enable them to overcome host iron-binding proteins and thrive in otherwise iron-limited environments (Budzikiewicz, 2001; Bauer *et al.*, 2002).

In Gram-negative bacteria the siderophore complex is internalised through an outer membrane channel that displays ligand specificity and energy dependency. Transport across the inner membrane is mediated by less specific ABC transporters. *Sodalis glossinidius* has been found to have putative siderophore biosynthesis, uptake and transport genes encoded on the largest of its extrachromosomal elements, pSG1 (Darby *et al.*, 2005). These genes code for an achromobactin-like (citrate) siderophore (*acsABCD, yhcA, lysA,* and *acr*: SGP1_0039 - SGP1_0045) and its ABC transporter (*cbrABCD*: SGP1_0035 – SGP1_0038). In addition, *S. glossinidius* encodes genes for a specific ABC transporter (SG1538 – SG1540) that is homologous to HutB/HemU/HmuV of *Yersinia enterolitica*. This would enable the direct transport of haem or haemoglobin across the cytoplasmic membrane via a specific ABC transporter (Toh *et al.*, 2006). *S. glossinidius* also has a chromosomally-encoded TonB-independent iron transport system.

3.1.5 Aims

The aims of the present work were to establish the growth dynamics, under *in vitro* axenic culture, for isolates of *S. glossinidius* from different species of *Glossina*. This information would allow the observation of conditions under which the growth of this bacterium is altered and show if there were differences in the growth dynamics of different *S. glossinidius* isolates. The conditions considered in the current work included incubation temperature, alteration of the constituents of the growth media and supplementation of the media with metal ions.

The iron metabolism was also investigated in the present work. Genes hypothetically involved in siderophore synthesis and transportation have been found on an extrachromosomal element of *S. glossinidius* (Darby *et al.*, 2005) and more recently on the *S. glossinidius* genome itself. The production of siderophores by *S.*

glossinidius was established using the chrome azurol S (CAS) assay as well as reverse transcription PCR. Siderophore production was also measured alongside the growth of the bacterial culture, in order to establish if siderophore production was constant or peaked at specific times.

3.2 Results

3.2.1 *Sodalis glossinidius* phenotype

Growth of *S. glossinidius* on MMI blood agar plates was achieved from all tsetse sources. Three isolates from each fly species were cloned by the standard bacterial practice of picking single colonies and streaking for clones (Figure 3.2). The colonies on MMI blood plates were visible within three days of plating and had grown to a diameter of greater than 1 mm by day four. Individual colonies up to 10 days old were 1 – 2 mm in diameter and were smooth, round and glossy. Colonies that were more than 10 days old became flattened and dull with a distinctive, irregular and undulating edge. Colonies of this second form gave rise to the first morphology when streaked onto fresh media.

Figure 3.2: *Sodalis glossinidius* clonal strain GA cultured on 10% packed horse blood Mitsuhashi and Maramorosch insect (MMI) medium agar (1%) in microaerobic atmosphere at 26.5°C (day 4 post-inoculation). Image reproduced from (Matthew *et al.*, 2005).

3.2.2 Growth kinetics of *Sodalis glossinidius*.

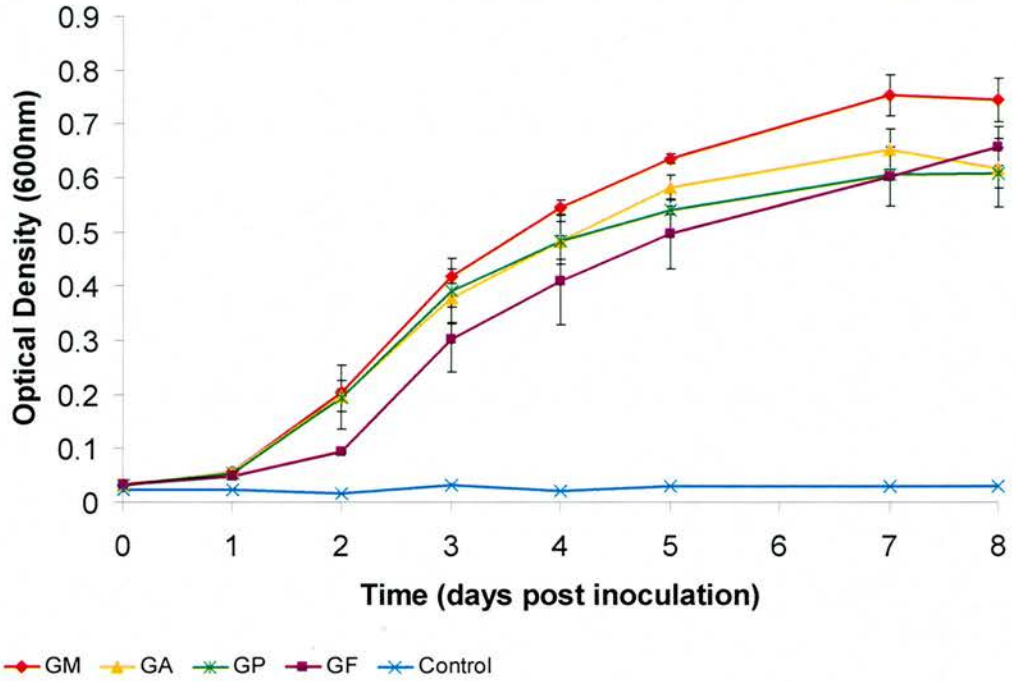
3.2.2.1 Growth of *S. glossinidius* isolated from different species of *Glossina*.

Growth curves were established for several isolates of *S. glossinidius*: GA, GM, GF and GP (Graph 3.1, Table 3.1). There was shown to be no significant difference in the rate of growth of these four isolates ($F_{3,86} = 0.194$, $p = 0.900$).

Strain Name	Species of *Glossina*
GM	*G. m. morsitans*
GA	*G. austeni*
GP	*G. p. palpalis*
GF	*G. f. fuscipes*

Table 3.1: Strain nomenclature for isolates of *Sodalis glossinidius* from different species of *Glossina*.

The lag phase of growth lasted for a mean of 30 hours, exponential phase for a mean of 90 hours and stationary phase occurred after six days of growth. The doubling time of *S. glossinidius* cultures was found to be 26 hours.



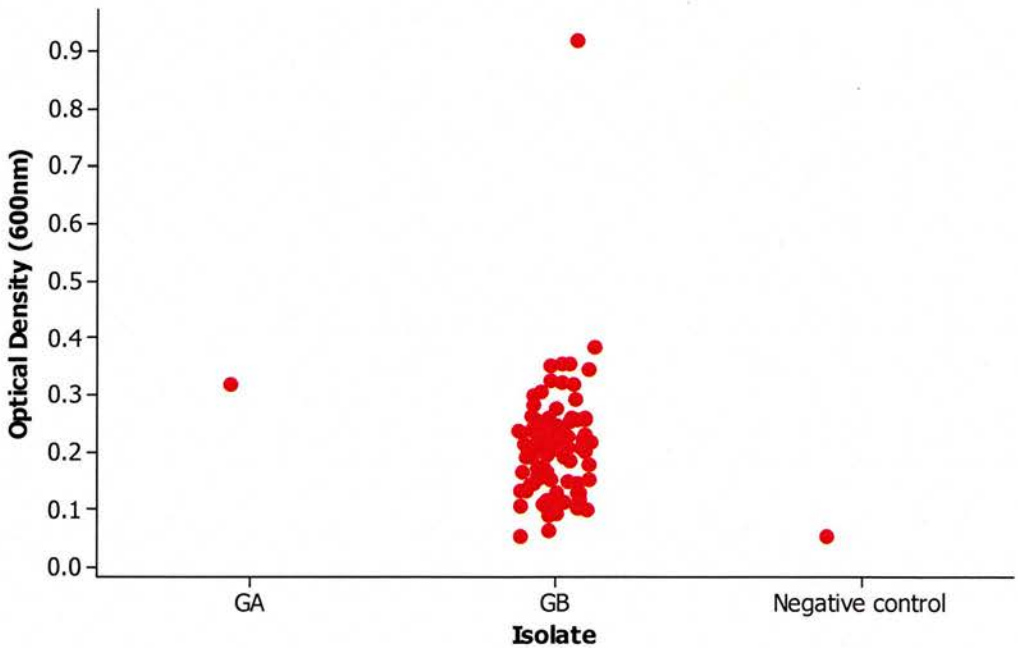
Graph 3.1: Mean growth curves of four strains of *Sodalis glossinidius* showing the optical density (600 nm) of the liquid culture against the time from inoculation ($n = 3$). Error bars show 95% confidence interval.

3.2.2.2 Differences in growth between *S. glossinidius* GB isolates

S. glossinidius was more difficult to isolate from *G. brevipalpis* than from the previous species of tsetse tested. Isolation required the accumulation of haemolymph from a number of individual flies, as opposed to the use of a single fly in *S. glossinidius* isolations from *G. m. morsitans* for example. The growth of *S. glossinidius* GB on solid medium showed a range of colony sizes and morphologies, suggesting that there might be differences between individual strains. For this reason, one hundred individual colonies were randomly chosen from the initial isolation and grown separately in order to assess differences in the rate of growth. Whilst some grew at a comparable rate to *S. glossinidius* isolated from other species

of *Glossina*, other isolates grew very slowly and sparsely, forming only a few small colonies on the plate.

The optical density (OD) of one hundred isolates of *S. glossinidius* GB was measured at 4 days post inoculation, when the culture was in the exponential phase of growth (Graph 3.2). The OD values ranged from 0.053 to 0.920, however, the latter value was an extreme outlier in the group. If the value of 0.902 were to be excluded as an anomaly, the range of OD values would be 0.053 to 0.384. As a comparison, the OD of *S. glossinidius* GA grown under the same conditions was 0.320. The negative control of uninoculated media had an OD of 0.053, suggesting that some of the *S. glossinidius* GB isolates had failed to grow in liquid culture.

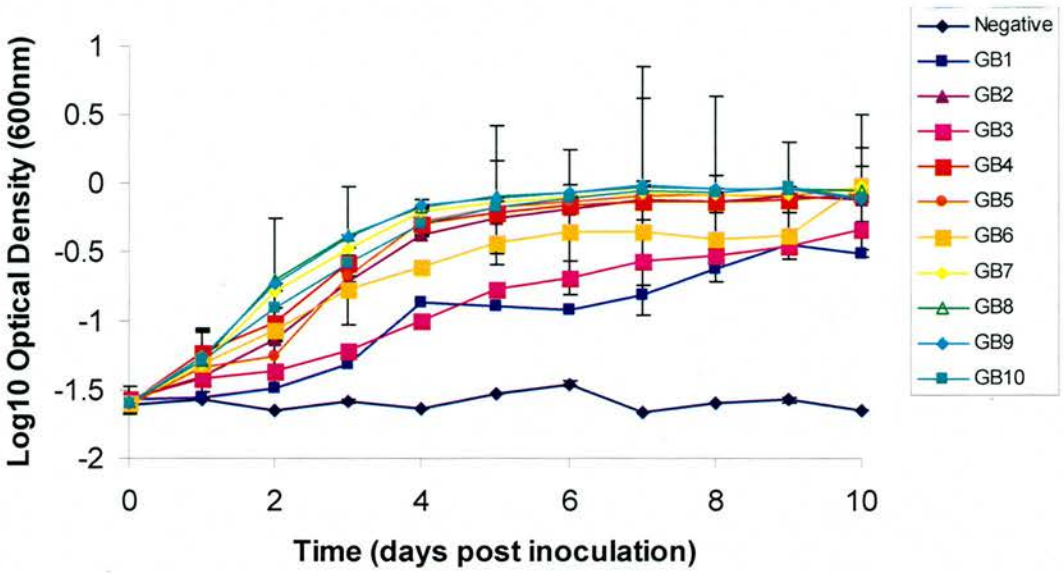


Graph 3.2: Optical density of 100 isolates of *S. glossinidius* GB at 4 days post-inoculation. *S. glossinidius* GA was included as a positive control. Uninoculated media was used as a negative control.

The dispersion of the OD data was tested using the index of dispersion. There was a significant difference of the variance:mean ratio to 1 so the data was not randomly distributed and patterns were formed, i.e. the isolates fall into separate groups, rather than covering a continuum of values between 0.053 and 0.384. This remained the case regardless of whether the anomalous data point was included (Index of dispersion = 4.56, $\chi^2_{99} = 451.3$) or excluded (Index of dispersion = 2.26, $\chi^2_{98} = 221.9$).

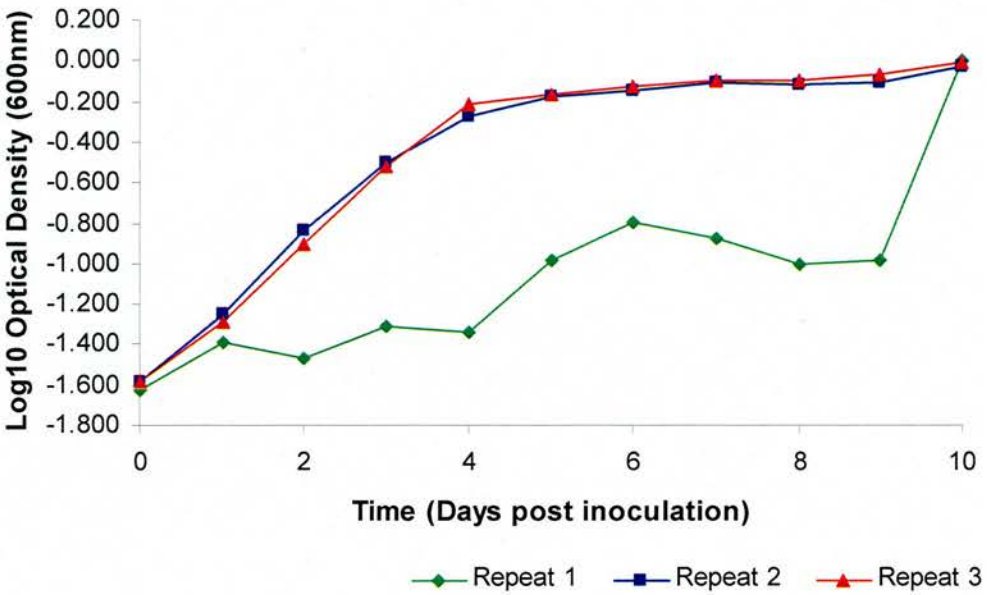
The growth curve of *S. glossinidius* GB was also established. Ten isolates were randomly chosen to give a representative sample of the one hundred original colonies. This gave a realistic number of bacterial cultures for optical density measurement when the experiment was conducted in triplicate.

There was a significant difference between the growth rates of the ten GB *S. glossinidius* isolates ($F_{9,80} = 4.426$, $p < 0.001$). The data was visually inspected to determine isolates which appeared to have different rates of growth from the majority. As can be seen in Graph 3.3, isolate GB3 appears to have a very different rate of growth. Isolate GB1 initially appears to have a similar rate of growth to GB3, but upon closer inspection it can be seen that the slope of the line, and therefore the rate of growth, between days 2 and 4 is more comparable to that of the other isolates, and the confounding factor is that GB1 appears to enter stationary phase relatively early.



Graph 3.3: Variation in growth curves of ten isolates of *S. glossinidius* GB over ten days. Error bars show the 95% confidence interval. Data points are the mean of three replicates.

While exclusion of isolate GB3 from the data set reduced the level of statistical significance markedly, there was still a significant difference ($F_{8,72} = 2.143$, $p = 0.043$) between the growth rates of the nine remaining isolates. Further examination of the data led to the exclusion of one of the three repeats of isolate GB6 as the growth in this replicate appeared to be anomalous in comparison to the other two repeats of isolate GB6 (Graph 3.4). If both GB3 and GB6.1 were excluded, then there was no significant difference between the growth rates of the remaining isolates ($F_{8,69} = 1.721$, $p = 0.109$).



Graph 3.4: The anomalous growth of one of three individual repeats of *S. glossinidius* isolate GB6 over ten days.

Overall, there was a significant difference between isolates in the lengths of the lag phases of the ten isolates ($F_{9,20} = 4.917$, $p = 0.002$). The isolates were found to fall into two groups in terms of lag phase length. The first group consisted of isolates GB1 and GB3, the intercepts of which were not significantly different ($F_{1,4} = 0.175$, $p = 0.698$). The remaining eight isolates formed the second group, the intercepts of which were not significantly different ($F_{7,15} = 1.656$, $p = 0.195$). The difference between the lag phases of these two groups was significant ($F_{1,28} = 33.11$, $p < 0.001$).

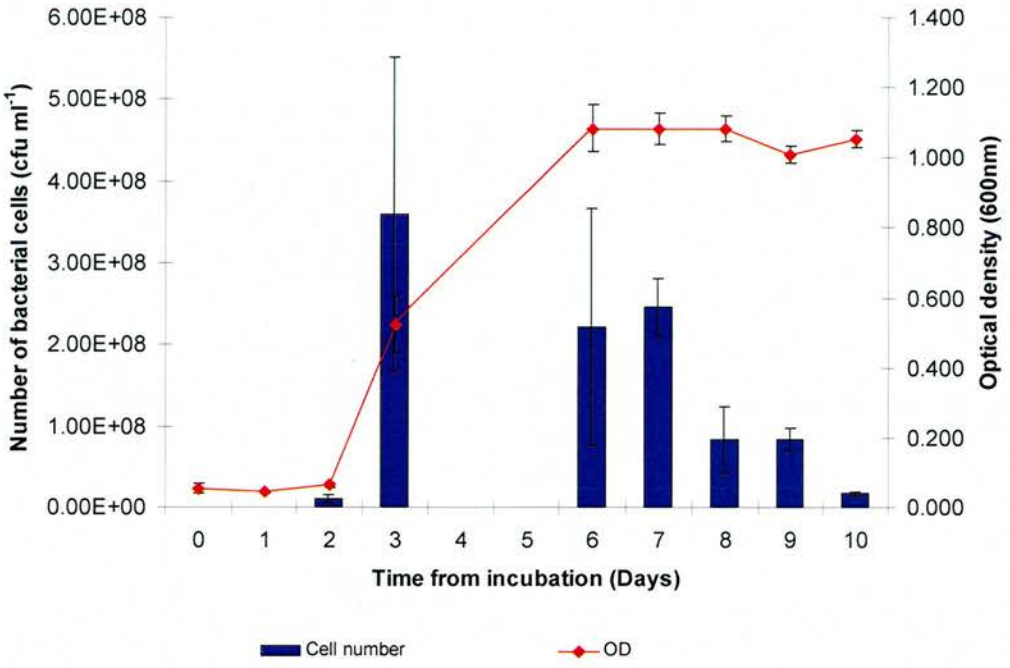
The generation time of the ten *S. glossinidius* GB isolates studied ranged from a minimum of 14 hours to a maximum of 32 hours (Table 3.2). The mean generation time for the ten isolates was 26 hours.

***S. glossinidius* GB isolate**	**Generation time (hours)**
GB1	14
GB2	29
GB3	32
GB4	24
GB5	20
GB6	32
GB7	21
GB8	32
GB9	30
GB10	23

Table 3.2: Generation time of ten *S. glossinidius* GB isolates.

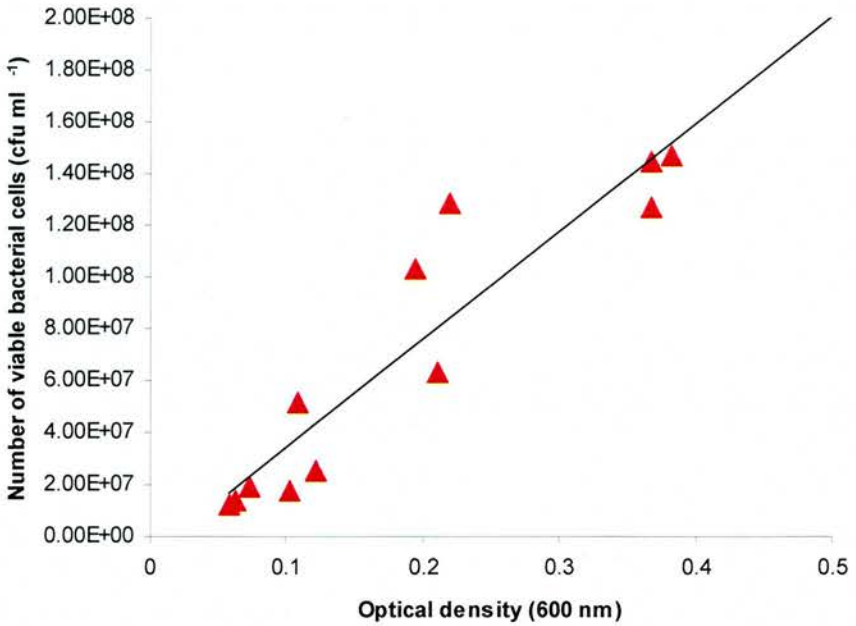
3.2.2.3 Viable cell counts and optical density measurements

The number of viable cells decreased markedly throughout the stationary phase of growth. The optical density of the culture stayed constant in stationary phase, even as the number of viable cells fell (Graph 3.5).



Graph 3.5: Viable cell counts of *S. glossinidius* GP in comparison with optical density measurements of the bacterial culture.

The optical density values of a serially diluted log phase culture of *S. glossinidius*, when compared to the number of viable bacteria in the dilutions, showed a positive relationship. Regression analysis of the number of viable cells per ml of bacterial culture showed that there was a significant correlation ($p < 0.001$, R-Sq = 86.8%) to the optical density (at 600 nm) of the culture (Graph 3.6).



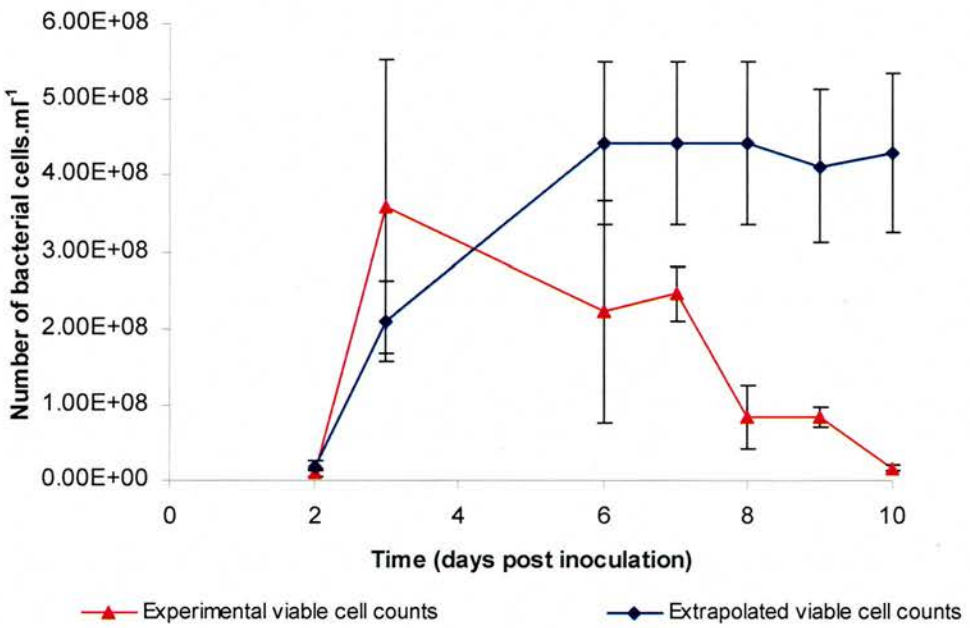
Graph 3.6: The number of viable cells per ml is related to the OD (600 nm) of liquid cultures of *Sodalis glossinidius*. The solid line is the best fit calculated by linear regression analysis.

The relationship between the optical density of the liquid culture of *S. glossinidius* and the number of viable cells per ml was used to calculate the number of cells present in later experiments (Equation 3.1).

$$y = 415727248x + 7489117$$

Equation 3.1: The relationship between optical density and the number of S. glossinidius cells, where y is the number of bacterial cells and x is the optical density of the bacterial culture at 600nm.

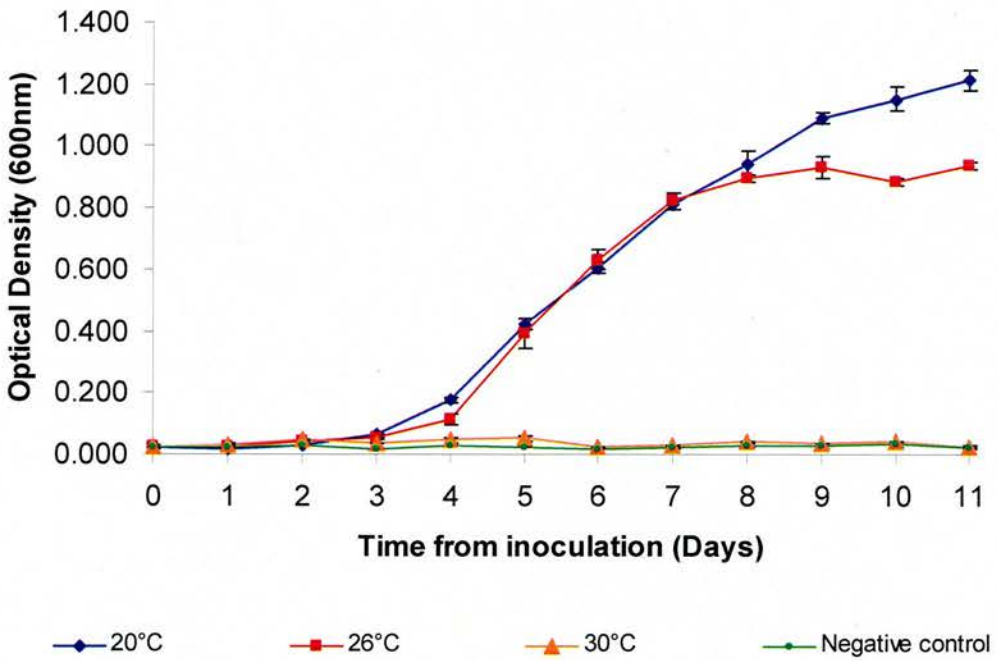
The number of bacterial cells, measured by viable cell counts of colony forming units (CFUs), was found to peak at three days post inoculation at 3.6 x 10^8 cells.ml^{-1}. This corresponds with the exponential phase of growth (Graph 3.5). The number of CFUs was seen to steadily decline after this point, as bacterial growth entered stationary phase, down to 1.67 x 10^7 cells.ml^{-1} at ten days post inoculation. The extrapolation of the relationship found between the number of viable cells.ml^{-1} and the optical density at 600 nm of liquid cultures of *S. glossinidius* (Equation 3.1) suggested that bacterial cell numbers would gradually increase during exponential growth phase and remain at a constant level of approximately 4.4 x 10^8 cells.ml^{-1} throughout stationary growth phase (Graph 3.7).



Graph 3.7: Comparison of viable cell counts of *S. glossinidius* GP with expected cell counts from calculations based on culture OD. Error bars show 95% confidence intervals.

3.2.2.4 Growth at different incubation temperatures

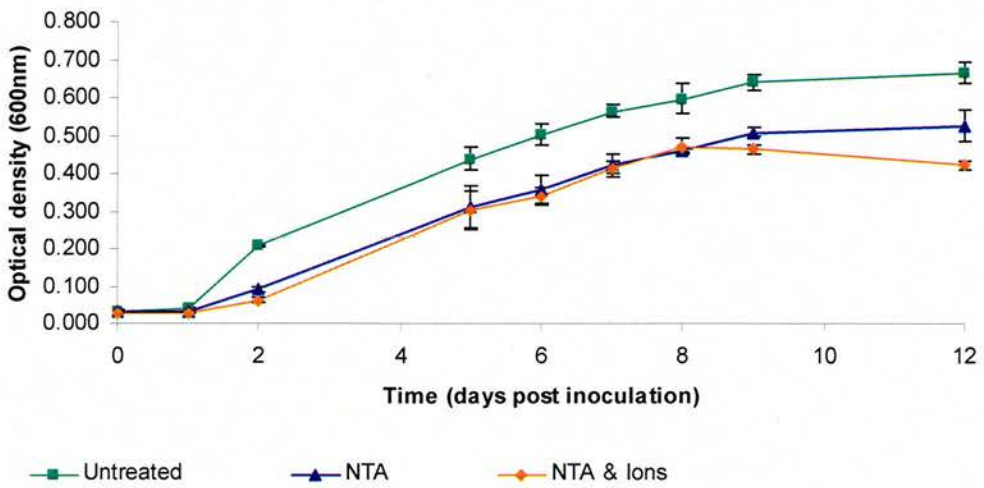
The growth of *S. glossinidius* was shown to be significantly inhibited at 30°C compared to growth at both 20°C and 26°C ($F_{2,24} = 39.89$, $p < 0.001$) (Graph 3.8). At 30°C there was no significant difference ($F_{1,10} = 1.33$, $p = 0.275$) between the inoculated culture and the negative control, an uninoculated culture, suggesting that growth of *S. glossinidius* was completely inhibited at this temperature. Growth at 20°C showed no significant difference from that at 26°C ($F_{1,16} = 2.73$, $p = 0.117$).



Graph 3.8: Growth of *S. glossinidius* GA at different incubation temperatures. Negative controls are the average of uninoculated controls incubated at each temperature (n = 3). Error bars show 95% confidence intervals. Data points represent the mean of three replicates.

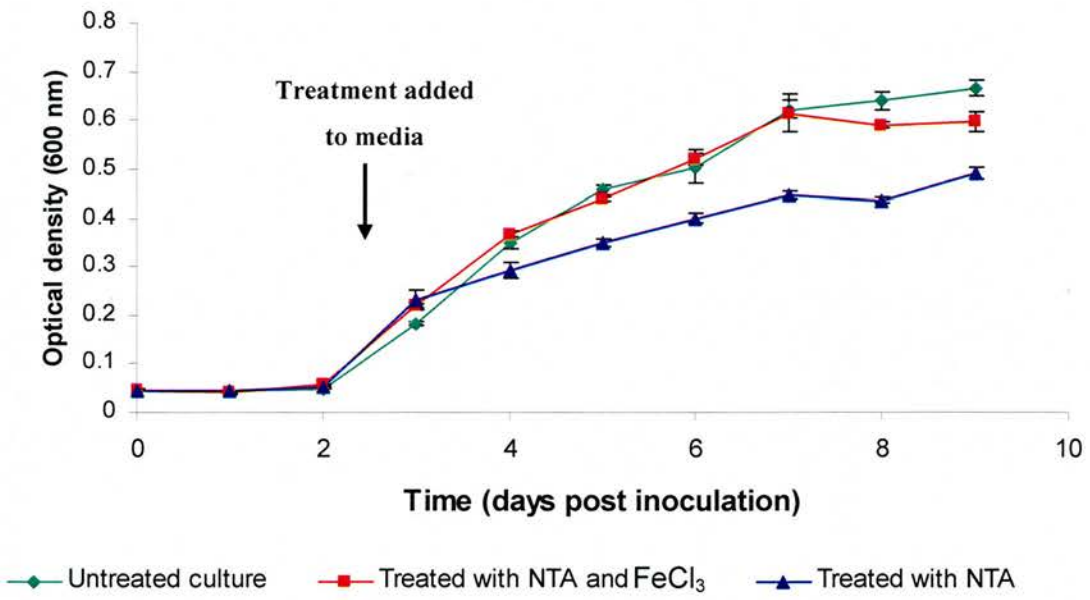
3.2.2.5 Growth with various concentrations of different chelating agents and with supplementary ions

Growth curves were measured to show the effect of different nutrient conditions in the medium on the growth of *S. glossinidius*. Growth media was rendered iron-deficient by the addition of the chelating agent, NTA (Graph 3.9). This was found to significantly reduce the rate of growth of *S. glossinidius* ($F_{1,28} = 10.451$, $p = 0.003$). Media treated with 5mM NTA was also supplemented with ions in order to verify that the reduction in bacterial growth was due to iron deficiency and not that of other metal ions, such as Zn^{2+}, Mg^{2+} and Mn^{2+}, which would also be chelated by the NTA. Growth of *S. glossinidius* was not restored by the addition of these ions and as such the growth rate showed no significant difference to that of *S. glossinidius* grown in the NTA-treated media ($F_{1,28} = 1.403$, $p = 0.246$) and was significantly lower than that of *S. glossinidius* grown in the untreated media ($F_{1,28} = 14.800$, $p < 0.001$).



Graph 3.9: Growth of *S. glossinidius* GP in media treated with a chelating agent and media subsequently supplemented with metal ions. Error bars show 95% confidence intervals. Data points represent the mean of three replicates.

In order to establish whether iron deficiency would affect the growth of bacteria in exponential phase the chelating agent, NTA, was added to the culture at day 3 of growth (Graph 3.10). Whereas prior to treatment there was no significant difference in the rates of growth of *S. glossinidius* in the three samples, on addition of the chelating agent the growth rate dropped significantly ($F_{1,22} = 39.770$, $p < 0.001$). The effect of the chelating agent on the growth of *S. glossinidius* was found to be negated by the simultaneous addition of iron to the medium, alongside the chelating agent ($F_{1,22} = 31.613$, $p < 0.001$). This treatment restored growth to normal levels, showing that iron is a limiting factor for the growth of *S. glossinidius* ($F_{1,22} = 0.803$, $p = 0.380$).

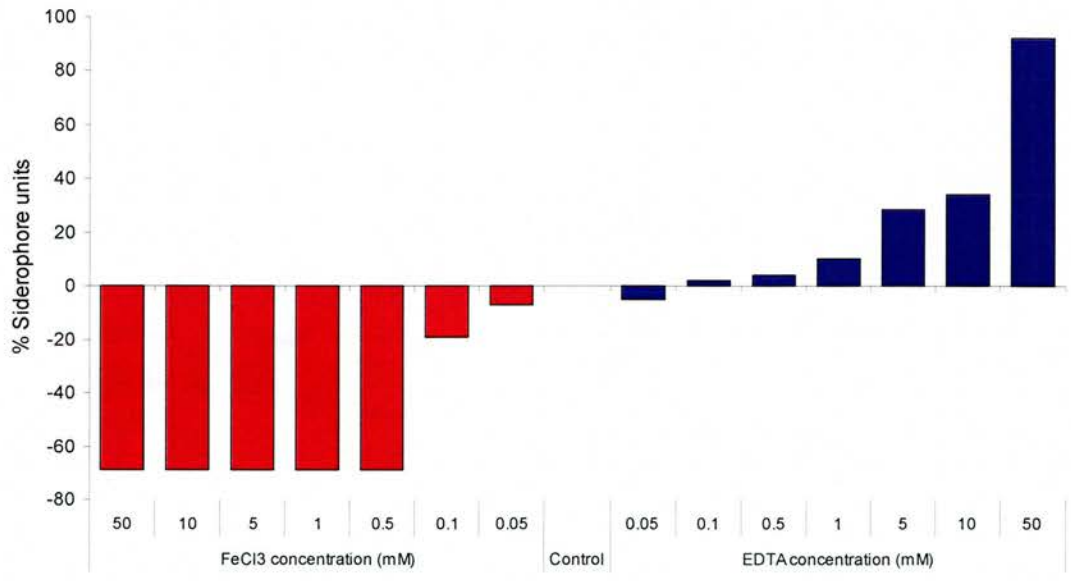


Graph 3.10: Growth of *S. glossinidius* GP in media treated with a chelating agent in log phase and media subsequently supplemented with $FeCl_3$. Error bars show 95% confidence intervals. Data points represent the mean of three replicates.

3.2.3 Chrome azurol S assays.

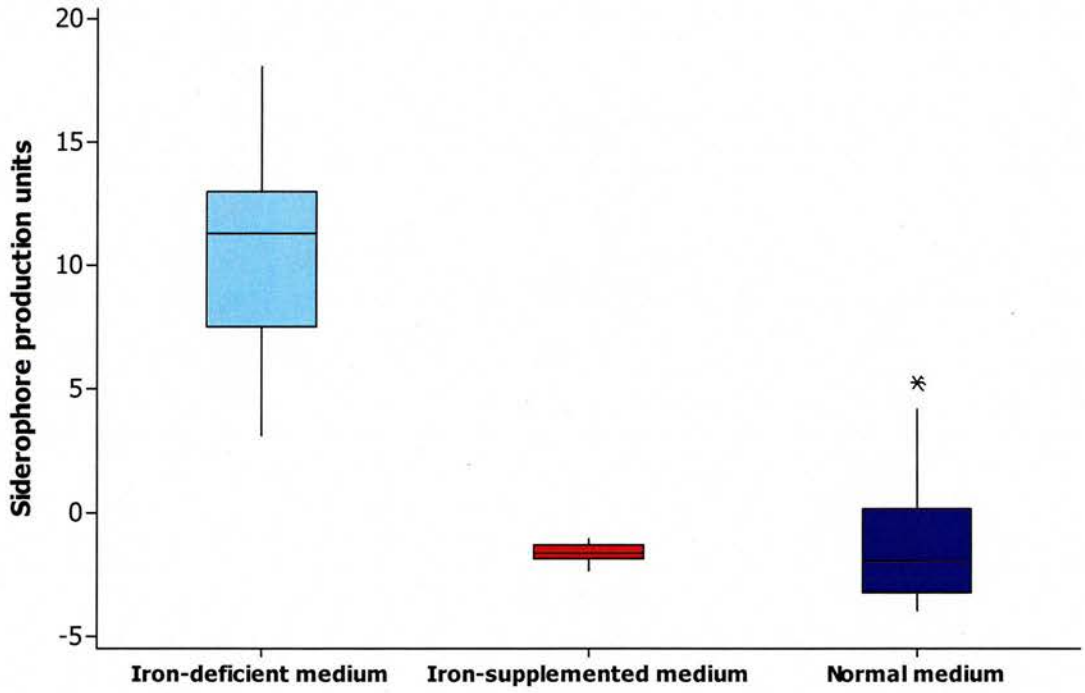
3.2.3.1 Quantification of siderophore production using liquid CAS

The CAS assay measures the ability of any siderophores present in the bacterial culture supernatant to bind iron from the indicator dye. This measurement is expressed in % siderophore units and is calculated using uninoculated medium as a control reference. This technique has been used to show the production of siderophores by *S. glossinidius* in iron-starved cultures and a 'standard curve' of colour change has been created using known concentrations of $FeCl_3$ and EDTA (Graph 3.11). This has confirmed that EDTA is a weaker chelating agent than both the proteins that sequester iron in MMI and the siderophore of *S. glossinidius.* This technique may be used to identify mutants in siderophore production – either those that under- or over-produce.



Graph 3.11: CAS liquid assay with known concentrations of EDTA and $FeCl_3$.

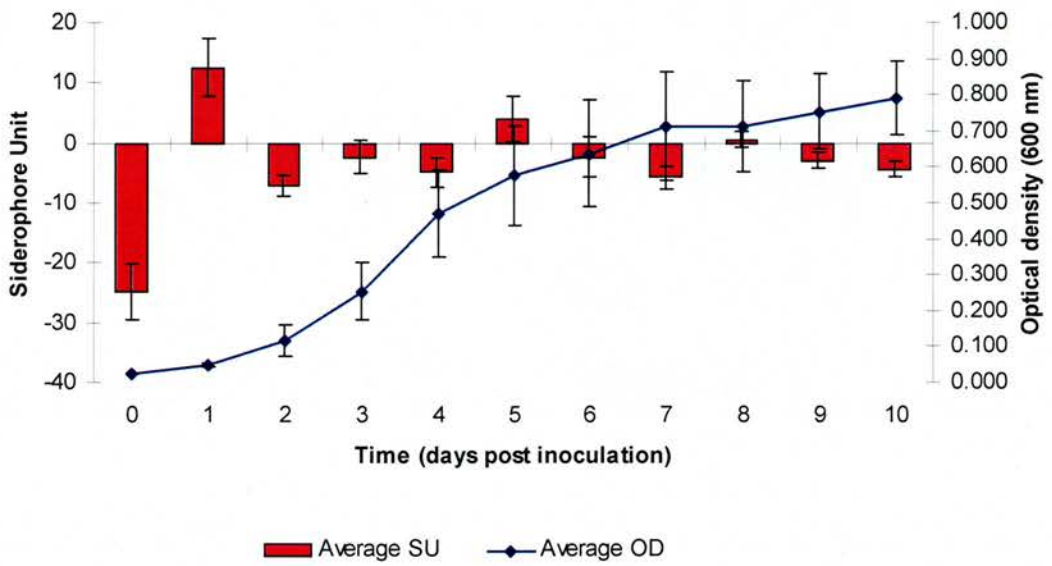
The production of siderophores by *S. glossinidius* was measured in normal growth medium and under conditions of iron-deprivation and iron-supplementation (Graph 3.12). In iron-deficient medium, *S. glossinidius* was found to produce significantly more siderophores than in either normal medium ($T = 10.60$, $p < 0.001$) or iron-supplemented medium ($T = 10.56$, $p < 0.001$). There was no significant difference in the production of siderophores in normal and iron-supplemented media ($T = 0.72$, $p = 0.487$).



Graph 3.12: Box plot of siderophore production units by *S. glossinidius* GA in iron-deficient, iron-supplemented and untreated medium. Outlying results are represented by an asterisk.

The optical density of *S. glossinidius* GB was measured alongside the siderophore production of the bacterial cultures (Graph 3.13). The growth curve of *S. glossinidius* GB followed the same pattern as isolates of *S. glossinidius* from

different species of *Glossina* with a generation time of 26 hours. Siderophore production was seen to peak at days 1, 5 and 8 during lag, late exponential and stationary phase respectively. The siderophore production units were compared between consecutive days using a two-tailed t-test (Table 3.3). The increase in siderophore production at day 1 was significant ($T = -11.66$, $p < 0.001$) as was the proceeding decrease at day 2 ($T = 7.37$, $p < 0.001$). The peak in siderophore production between days 4 and 5 was also significant ($T = -4.54$, $p = 0.001$) as was the peak between days 7 and 8 ($T = -4.51$, $p = 0.001$). There were also significant decreases in siderophore production by *S. glossinidius* GB between days 5 and 6 ($T = 2.62$, $p = 0.028$), 8 and 9 ($T = 4.01$, $p = 0.003$) and 9 and 10 ($T = 2.73$, $p = 0.023$).



Graph 3.13: Siderophore production and growth of *S. glossinidius* GB. Error bars show 95% confidence intervals. Data points represent the mean of three replicates.

Day comparison	T-Value	P-Value	Significant change
0 and 1	-11.66	<0.001	Increase
1 and 2	7.37	<0.001	Decrease
2 and 3	-2.81	0.020	Increase
3 and 4	2.73	0.023	Decrease
4 and 5	-4.54	0.001	Increase
5 and 6	2.62	0.028	Decrease
6 and 7	1.78	0.109	-
7 and 8	-4.51	0.001	Increase
8 and 9	4.01	0.003	Decrease
9 and 10	2.73	0.023	Decrease

Table 3.3: Paired t-test of siderophore unit production by *S. glossinidius* GB between consecutive days.

3.2.3.2 Qualitative determination of siderophore production using CAS agar.

Although *S. glossinidius* grows very poorly on CAS agar, it is possible to visualise siderophore production by the colour change around bacterial colonies (Figure 3.3). Siderophore production was shown for each of the three *S. glossinidius* isolates. No colour change was seen when CAS agar was inoculated with heat-killed *S. glossinidius*.

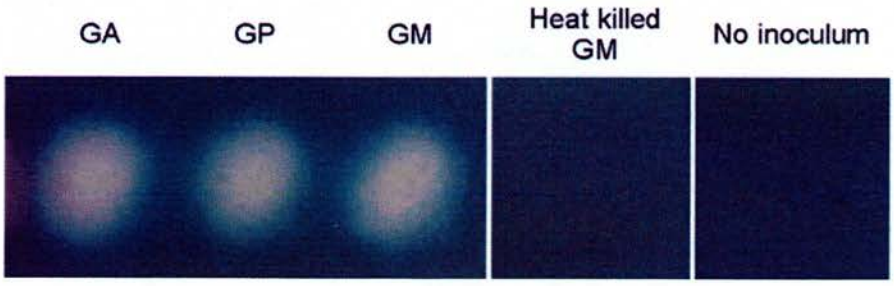


Figure 3.3: Visualisation of siderophore production by *S. glossinidius* isolates GA, GP and GM on CAS iron indicator agar. A colour change from blue to yellow takes place when iron is removed from the medium. Image reproduced from (Darby *et al.*, 2005).

3.2.3.3 Reverse transcription of log-phase bacterial cultures for siderophore gene expression

The expression of the gene *P1 103* from the extrachromosomal element pSG1 was shown by the reverse transcription of RNA from log-phase cultures of *S. glossinidius* (Figure 3.4). After gel electrophoresis, bands of 110bp were apparent for each *S. glossinidius* isolate tested (GA, GM and GP).

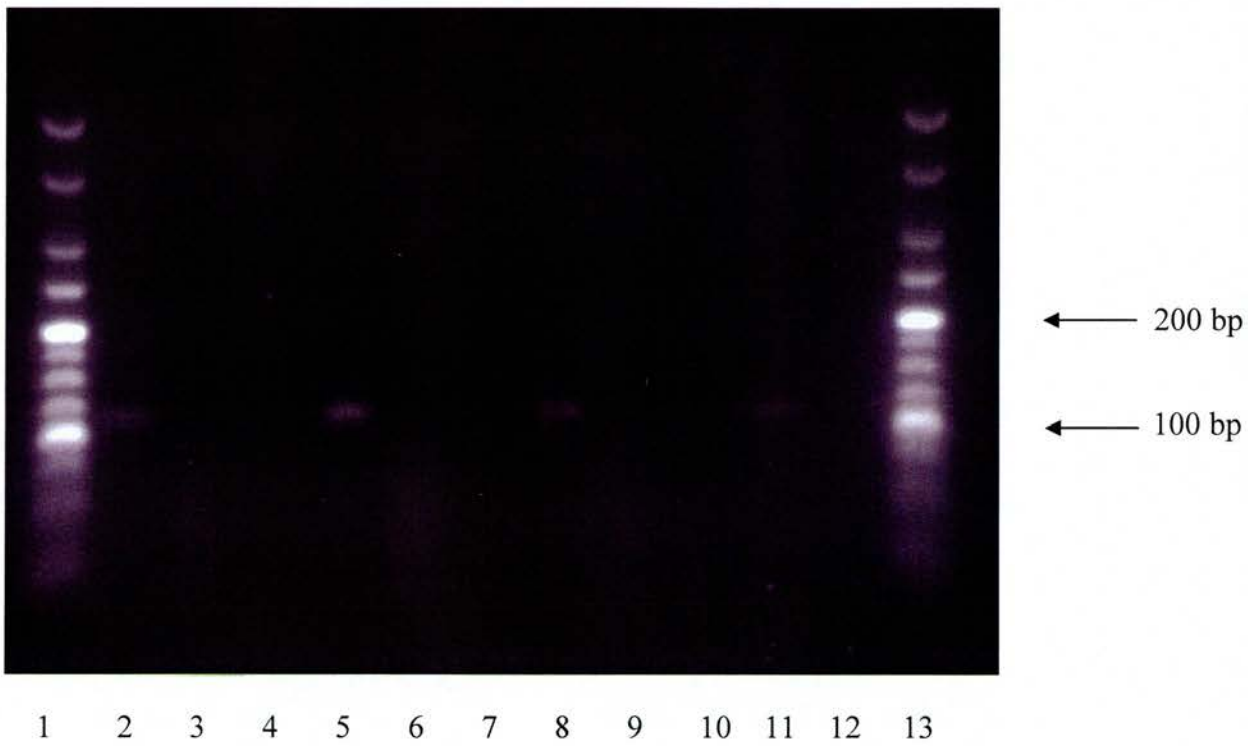


Figure 3.4: Gel electrophoresis of PCR for *P1 103* primers on bacterial growth from log phase *S. glossinidius* bacterial cultures. Lanes 1 and 13 – 25 bp molecular weight marker; lanes 2, 5 and 8– *S. glossinidius* GA, GM and GP (respectively) cDNA; lanes 3, 6 and 9 - *S. glossinidius* GA, GM and GP (respectively) RNA ; lanes 4, 7, 10 and 12 – H_2O negative control; lane 11 – pSG1 DNA positive control.

3.3 Discussion

3.3.1 Studies into the growth of *S. glossinidius*

3.3.1.1 Growth kinetics of *Sodalis glossinidius*

The growth curve of *S. glossinidius* follows a standard pattern of lag phase, exponential phase and stationary phase, although each of these is extended over a period of days as the bacteria have a relatively slow growth rate in comparison to others, such as *Escherichia coli*, which has a doubling time of 20 minutes. Linear mixed effect models were used to analyse the growth curve data during exponential phase. This exclusion of data from lag and stationary phase was necessary for the statistical analysis but disregarded the initial lag phase of the bacteria, which may account for the variance in the final optical densities of the bacterial cultures.

There was no significant difference between the rate of growth of *S. glossinidius* isolated from *G. austeni*, *G. f. fuscipes*, *G. m. morsitans* or *G. p. palpalis*. This was not an unexpected result as 16S rDNA based phylogenetic analysis of *S. glossinidius* has shown very high levels of homology between isolates from different *Glossina* species (Aksoy *et al.*, 1997). *S. glossinidius* GB was found to exhibit a range of growth rates between individual isolates, the mean of which was 26 hours. This was the same mean generation time as that found for *S. glossinidius* GA, GF, GM and GP strains. Some *S. glossinidius* GB isolates were apparently unable to grow in liquid medium, possibly helping to explain the initial difficulties experienced in isolating *S. glossinidius* from *G. brevipalpis*. The necessity of combining the haemolymph of numerous flies in order to isolate *S. glossinidius* from *G. brevipalpis*, when one fly is sufficient with the other species of *Glossina* used, may be a result of the lower population numbers of *S. glossinidius* in *G. brevipalpis*. *G. brevipalpis* was found to

have significantly lower *S. glossinidius* levels than *G. m. morsitans* or *G. p. palpalis* in semi-quantitative measurements by Cheng and Aksoy (Cheng & Aksoy, 1999). However, the same study found *G. austeni* to have similarly low symbiont levels and yet there were no difficulties experienced in isolating *S. glossinidius* from this species of tsetse.

The growth dynamics of *S. glossinidius* have previously been studied in cell culture (Welburn, 1991). The bacteria were grown in an *Aedes albopictus* cell monolayer and the number of bacteria per cell and the proportion of host cells infected were measured over a period of time. These studies found that there was a significant difference between the generation time of *S. glossinidius* derived from different species of tsetse. The generation times ranged from 4.1 hours (*G. pallidipes*) to 13.6 hours (*G.tachinoides*). The generation time of *S. glossinidius* isolates from *G. austeni*, *G. m. morsitans* and *G. p. palpalis* were very similar to each other and ranged from 4.24 to 5.85 hours. No *S. glossinidius* isolates from *G. brevipalpis* were included in these previous studies.

The present work found no significant difference in the growth dynamics of *S. glossinidius* strains isolated from different species of *Glossina*, although the axenic growth conditions were very different to the cell culture method used by Welburn (1991). This may help to explain the fact that the generation times of *S. glossinidius* found by Welburn (1991) ranged between 4.1 to 13.6 hours whilst the present work found *S. glossinidius* to have a generation time of 26 hours. However, the study by Welburn (1991) included a wider variety of strains of *S. glossinidius* isolated from different species of *Glossina* and the species which were included in the present work all displayed very similar generation times. This suggests that the conclusions drawn by the present work, that the generation times of different strains of *S. glossinidius* are not significantly different, may not be more broadly applicable to strains of *S. glossinidius* isolated from other tsetse species.

3.3.1.2 Growth temperature of *Sodalis glossinidius*

The growth of *S. glossinidius* is optimal at temperatures lower than usual for mesophilic bacteria. This is likely due to the body temperature of its host, the tsetse fly, which is approximately 26°C. The present work found the optimal temperature for the growth of *S. glossinidius* to be 26°C, with little or no growth seen at 30°C. This is in accordance with Dale and Maudlin who found an optimal temperature of 25°C with little or no growth seen at 30°C, using *S. glossinidius* strain M1 isolated from *G. m. morsitans* (Dale & Maudlin, 1999). Under laboratory conditions tsetse have been seen to become sterile when maintained at 30°C, an effect possibly attributable to the death at this temperature of the primary symbiont, *Wigglesworthia glossinidia* (Mellanby, 1936). In order to better understand the effect of high incubation temperatures on *S. glossinidius* it would be interesting to take a culture that had been incubated at 30°C for a period of time and study whether growth was restored if the culture were then to be incubated at 26°C.

More controversially, the present work found that there was no significant retardation of growth of *S. glossinidius* at 20°C. In the current study, *S. glossinidius* incubated at 20°C had a generation time of 34 hours, four hours longer than when the bacterium was incubated at 25°C. The reduction of temperature to 22°C has previously been used to slow the growth of *S. glossinidius in vitro* (Welburn *et al.*, 1987; Welburn & Gibson, 1989) and to lower *S. glossinidius* population numbers *in vivo* (Welburn & Maudlin, 1991). The generation time of *S. glossinidius* (*G. m. morsitans*) in *A.albopictus* cell culture was found to be significantly affected by the temperature of incubation. Whilst at 25°C the generation time was 5.4 hours, lowering the incubation temperature to 22°C increased the generation time six-fold to 32 hours (Welburn, 1991).

Previous studies *in vitro* were, however, carried out using insect cell lines in which to culture the bacteria whereas in the present work culture techniques have been optimised so that *S. glossinidius* may be grown axenically. Therefore the retardation of *S. glossinidius* growth under lower temperatures seen by Welburn *et al.* (1987) may have been at least partially attributable to the effect of the lower temperature on the insect cell line rather than the bacterium.

Environmental temperature is known to effect host-symbiont interactions (Thomas & Blanford, 2003). The density of *Wolbachia* spp. populations in the wasp *Leptopilina heterotoma* was found to be significantly altered by relatively small changes in temperature (Mouton *et al.*, 2006). In the Mouton *et al.* study, the optimal temperature for the growth of symbiont and host was different, with highest densities of *Wolbachia* spp. reached at 26°C. This difference may reflect an effect of higher temperatures on the ability of the host to regulate its symbiont population numbers (Mouton *et al.*, 2006).

Secondary endosymbionts have also been found to benefit their hosts under heat stress, as is the case in *Acyrthosiphon pisum* (Montllor *et al.*, 2002). In some cases it might be possible for the host to influence its own symbiont interactions by using behavioural thermoregulation as a means of regulating its symbiont population. Behavioural thermoregulation is the management of body temperature through behaviour such as seeking or avoiding sources of heat (Thomas & Blanford, 2003). The desert locust, *Schistocerca gregaria*, for example is able to raise its body temperature in response to fungal infection. Experiments have shown that this ability to induce fever can increase the lifespan and reproductive capability of locusts compared to those that have been stopped from entering fever (Elliot *et al.*, 2002).

3.3.2 Iron metabolism of *Sodalis glossinidius*

3.3.2.1 Growth dynamics of *Sodalis glossinidius* in iron-deficient conditions

The growth of *S. glossinidius* was found to be affected by the concentration of iron in the growth medium. In iron-depleted conditions, after treatment of the growth medium with a chelating agent, the growth rate of *S. glossinidius* was found to be significantly reduced. This is a common characteristic of bacterial growth under low iron concentration conditions, with the resultant inhibition of growth having been recorded in many bacteria, including *Mycobacterium avium* (Gomes *et al.*, 1999) and *Staphylococcus epidermidis* (Matinaho *et al.*, 2001). The effect of the chelator-treated medium on the growth of *S. glossinidius* was shown to be due to the reduction in iron concentration, rather than the chelation of other metal ions present in the medium, as the subsequent supplementation of the growth medium with manganese, magnesium and zinc ions had no restorative effect on the growth rate. The effect of the chelating agent on the growth of *S. glossinidius* was found to be negated by the simultaneous addition of iron to the medium, alongside the chelating agent. This treatment restored growth to normal levels, showing that iron is a limiting factor for the growth of *S. glossinidius*.

The colour change of the CAS agar shows that *S. glossinidius* does produce siderophores to chelate iron (Darby *et al.*, 2005). This is supported by both the liquid CAS assays and the reverse transcription of *S. glossinidius* RNA performed in the present work. The gene *P1 103*, which encodes for siderophore biosynthesis on plasmid pSG1, was found to be expressed in each of the three isolates of *S. glossinidius* analysed (GA, GM and GP).

Analysis of the percentage siderophore units produced by *S. glossinidius in vitro* has shown that the presence of siderophores in the culture supernatant changes over time. This may be due to reduced expression of the relevant genes during times when the bacterium is not under iron-stress. Peaks of siderophore production were seen at one day post inoculation, in lag phase, at 5 days post inoculation, in late exponential phase, and at 8 days post inoculation, in stationary phase. The initial peak in the lag phase of growth may relate to increased demand for iron by the bacterial cell as it actively metabolises in preparation for cell division. Peaks in late exponential and stationary phase may reflect the stress placed on the bacterial culture as increasing population numbers cause nutrients to become limiting. Stationary phase is also linked to a decrease in viable cells present in the culture. *S. glossinidius* has, however, been found not to have the global ferric uptake regulator (*fur*) genes, suggesting that regulation of siderophore gene expression must take place by another method (Toh *et al.*, 2006).

The range of percentage siderophore units measured in the present work ranged from negative values to positive values. This was unexpected, as negative values imply that the indicator dye is being loaded with iron, as opposed to having iron removed by chelating agents. The source of such iron is undetermined, but these results may be due to the complex nature of the media necessary for the growth of *S. glossinidius* (Shin *et al.*, 2001). In this case it may be more useful to assign relative values to the siderophore production units, as comparison of siderophore production rather than quantification is the aim.

When siderophore production was compared between cultures of *S. glossinidius* grown in media treated with a chelating agent, supplemented with iron or left untreated, significant differences were found between the three treatments. The production of siderophores by *S. glossinidius* grown in chelated media was significantly higher than that found in either iron supplemented or untreated media. This is in accordance with the hypothesis that siderophores would be selectively

produced by *S. glossinidius* under conditions of iron stress. Although there was a wider range of siderophore units found in untreated media than in iron-supplemented media, there was no significant difference between the two, indicating that untreated media is sufficiently iron-rich for the growth of *S. glossinidius* without the need for iron-scavenging behaviour by the bacterium.

Chapter 4

Quantification of *S.glossinidius* symbionts

4 Quantification of *Glossina* spp. Symbionts

4.1 Introduction

4.1.1 Development of Quantitative PCR

In the early 1990s Higuchi and colleagues began to develop a technique that made it possible to detect amplification products of the polymerase chain reaction as it progressed without the need to take aliquots. Ethidium bromide was included in the reaction mixture and the emitted fluorescence was measured with a fluorimeter coupled to a thermal cycler (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993). Today, more advanced intercalating agents, such as SYBR green are available, as well as product-specific fluorogenic probes. Quantitative data are derived from the determination of the reaction cycle at which the fluorescent signal from the product rises above a detection threshold. The number of this cycle is proportional to the amount of the target in the starting material (Heid *et al.*, 1996). Recent advances in this technique, including efficient detection chemistries and highly sensitive instruments have resulted in a broad range of detection sensitivity, spanning six logs as opposed to the two log range of traditional PCR, and the ability to detect a single DNA fragment (Kubista *et al.*, 2006).

4.1.2 Quantitative PCR in the elucidation of symbioses

Quantitative PCR (qPCR) has been used to analyse bacterial populations in many complex environments. Bacterial symbionts of aphids, filarial nematodes and mosquitoes, to mention but a few examples, have been quantified using qPCR

yielding information on the dynamics of the relationship and the interactions between various species of symbionts within a host. (Dutton & Sinkins, 2004; Fenn & Blaxter, 2004; Sakurai *et al.*, 2005).

The population size of symbionts within a host covers a broad range. Populations of *Wolbachia* spp. have been quantified in numerous different host insect species using different methods. The eggs of the minute wasp (genus *Trichogramma*) have been found to contain between 250 – 670 bacterial cells per egg using microscopy (Stouthamer & Werren, 1993). Eggs of *Drosophila simulans* (Riverside, California) have been found to contain up to 500,000 bacterial cells per egg using dot blots (Bourtzis *et al.*, 1996).

4.1.3 Quantitative PCR and the symbionts of *Glossina* spp.

The quantitative analysis of *S. glossinidius* populations in *Glossina* spp. was recommended by Welburn *et al.* as early as 1987, in order to further examine the nature of the association between tsetse susceptibility to trypanosome infection and *S. glossinidius* population and to find ways by which this association might be exploited for the control of African trypanosomiasis (Welburn *et al.*, 1987).

Quantitative PCR has enabled the accurate quantification of the bacterial populations in the tsetse fly, whereas previously more qualitative methods were used. Moloo and Shaw made qualitative judgements on the density of bacterial populations in different species of tsetse by microscopy, finding that teneral flies "invariably had fewer RLOs during the puparial development than either the 30- or 60-day old tsetse" (Moloo & Shaw, 1989).

Welburn and Maudlin (Welburn & Maudlin, 1991) quantified the *S. glossinidius* populations in *Glossina* spp. using both DNA probes and conventional staining. Homogenised tsetse midguts were spotted onto immunofluorescence slides which were fixed and stained using Gimenez staining. The slides were examined using light microscopy and a semi-quantitative measurement of *S. glossinidius* population size was made by determining the dilution end-point at which the bacteria could no longer be detected.

A semi-quantitative approach was also taken by Cheng and Aksoy, who classified the intensity of the PCR-amplification product based on the ethidium bromide staining (Cheng & Aksoy, 1999). Their analysis found that there were significantly lower densities of *S. glossinidius* infection in *G. austeni* and *G. brevipalpis* than were found in *G. m. morsitans* or *G. p. palpalis* (Cheng & Aksoy, 1999). These results were partly supported by the qualitative judgements made by Moloo and Shaw using microscopy (Moloo & Shaw, 1989).

4.1.4 Role of *S. glossinidius* in tsetse susceptibility to trypanosome infection

The prevalence of *S. glossinidius* in wild populations of tsetse flies has been of particular interest since Maudlin (Maudlin, 1982) established that tsetse susceptibility to midgut infection by trypanosomes is a maternally inherited characteristic which was later found to be associated with the presence of RLOs in the fly (Maudlin & Dukes, 1985; Maudlin & Ellis, 1985). The relationship between this bacterium and trypanosome infections of tsetse has been investigated in several studies (Maudlin *et al.*, 1990; Welburn *et al.*, 1993).

It is thought that the action of a chitinase, produced by *S. glossinidius*, breaks down chitin during pupal development, causing the build up of *N*-acetyl-D-glucosamine (Welburn *et al.*, 1993). This compound acts as an inhibitor of tsetse lectins, which play a role in the prevention of trypanosome infection in the fly (Maudlin & Welburn, 1987).

The presence of large numbers of micro-organisms in the midgut epithelial cells of *G. m. morsitans* infected with *Trypanosoma brucei* was reported by Hecker and Moloo (Hecker & Moloo, 1981), while Maudlin and Ellis (Maudlin & Ellis, 1985) found that lines of *G. m. morsitans* that were susceptible to *T.congolense* had a 90% prevalence of *S. glossinidius* infection whilst refractory lines had a prevalence of only 18%. The conclusions of a later study by Moloo and Shaw disagreed with the hypothesis that there was a simple causal relationship between *S. glossinidius* infection and susceptibility to trypanosomes, finding that all flies were positive for *S. glossinidius* regardless of their trypanosome-infection status (Moloo & Shaw, 1989). However, given that any influence of *S. glossinidius* on the refractoriness of the fly is likely to be proportional to the bacterium's population size within the fly, and that bacterial populations are dynamic and change over time, conclusions drawn from bacterial populations observed 30 days after the critical event of infection are not necessarily relevant.

4.1.5 Aims

In the present work the population of *S. glossinidius* was quantified over the life cycle of the tsetse fly, from pupa to five weeks post eclosion. Specific developmental stages of particular interest, namely newly deposited pupae and teneral flies, were examined in more detail using larger sample group sizes and both *G. m. morsitans* and *G. brevipalpis*. The technique of qPCR was also exploited to

look at the copy numbers of extrachromosomal elements associated with *S. glossinidius* both *in vitro* and *in vivo*.

Given the suggested link between *S. glossinidius* infection and susceptibility to trypanosomes, the populations of *S. glossinidius* were quantified in insects challenged with trypanosomes. Since any symbiont-associated effect is thought to be limited to the teneral state, a comparison of *S. glossinidius* populations in teneral and fed *G. m. morsitans* and *G. brevipalpis* was made.

Investigations into the density of *Glossina* spp. symbiont populations *in vivo* have recently been made by Rio et al. (Rio *et al.*, 2005). The findings of that study appear to come to different conclusions, as to the dynamics of growth of *S. glossinidius*, to those presented here. The possible reasons for these discrepancies are addressed in the discussion.

4.2 Results

4.2.1 The number of copies of the *SodGroEL* gene within a cell of *S. glossinidius*

The number of copies of the *SodGroEL* gene within a cell of *S. glossinidius* was established by using total cell counts alongside quantitative PCR (Figure 4.1). The quantitative analysis of the number of copies of *SodGroEL* within the sample enabled the calculation of the number of copies of the *SodGroEL* gene within a cell of *S. glossinidius* by using the mean of the number of cells. It was found that there are approximately 8 copies of the *SodGroEL* gene within a cell of *S. glossinidius*.

Total cell counts (cells/ml)	qPCR for copies of *SodGroEL* (per ml)	Copies of *SodGroEL* per cell
5.9×10^6	4.43×10^7	7.5

Figure 4.1: The number of copies of the *SodGroEL* gene within a cell of *S. glossinidius*. Total cell counts measure the number of *S. glossinidius* cells in the sample and qPCR was used to quantify the number of copies of *SodGroEL* in the sample. Data shown is a mean of three replicates.

Blastn analysis of the complete *S. glossinidius* genome showed that *SodGroEL* is a single copy gene so these results suggest that there are multiple genomes within a single *S. glossinidius* cell.

4.2.2 The pupal development of the tsetse fly

The development of *G. m. morsitans* pupae was investigated through dissection and microscopy to enable the comparison of the quantification of the tsetse's bacterial population with the developmental stage of the pupa (Figure 4.2). One day after deposition, it was not possible to separate the pupa from its case. There was apparently no development at this stage, with no physiological characteristics being observed under microscopy. By 10 days post deposition, formation of the head was visible and by 17 days, the pupae had clearly formed three divisions of head, thorax and abdomen and the eyes were apparent. At 20 days post deposition, the segmentation of the pupal body had become more pronounced and the legs had begun to form. Four days later, at 24 days post deposition, wings had clearly formed and over the next seven days a fully recognisable fly rapidly formed. Several individuals from the group sampled at 31 days post deposition had already undergone eclosion.

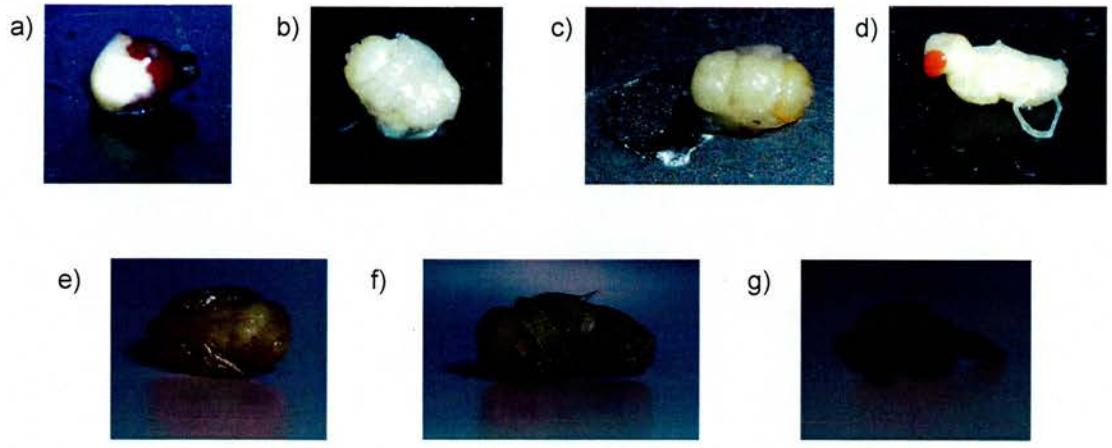
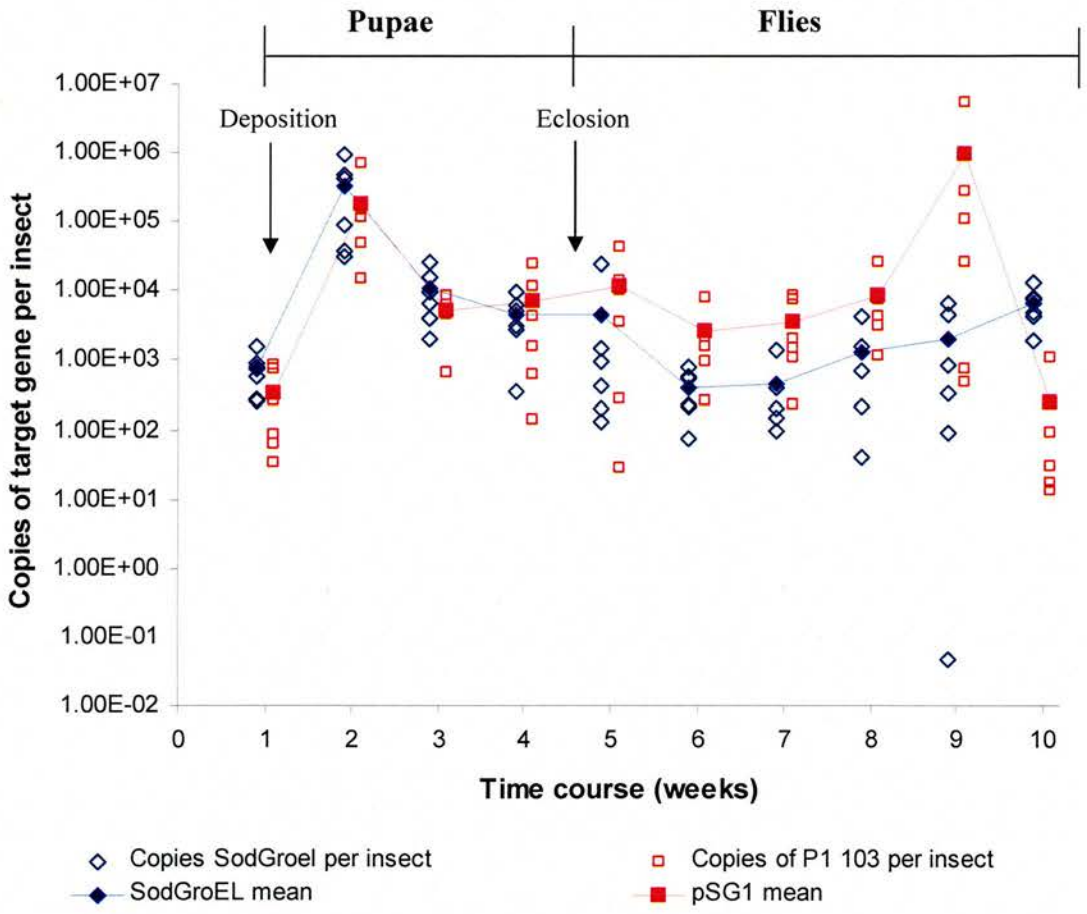


Figure 4.2: Tsetse pupal development. Pupae were dissected out from their cases and photographed at: a) 1 day post deposition, b) 10 days, c) 17 days, d) 20 days, e) 24 days, f) 27 days and g) 31 days.

4.2.3 Quantitative PCR of *S. glossinidius* and pSG1 in *G. m. morsitans* throughout the developmental course from pupa to adult

The number of copies of *SodGroEL* and *pSG1* in tsetse was measured throughout the developmental course of tsetse, from pupa to adult (Graph 4.1). Six individuals were randomly sampled at weekly intervals and qPCR was used to calculate the copy number of the target genes in each individual.



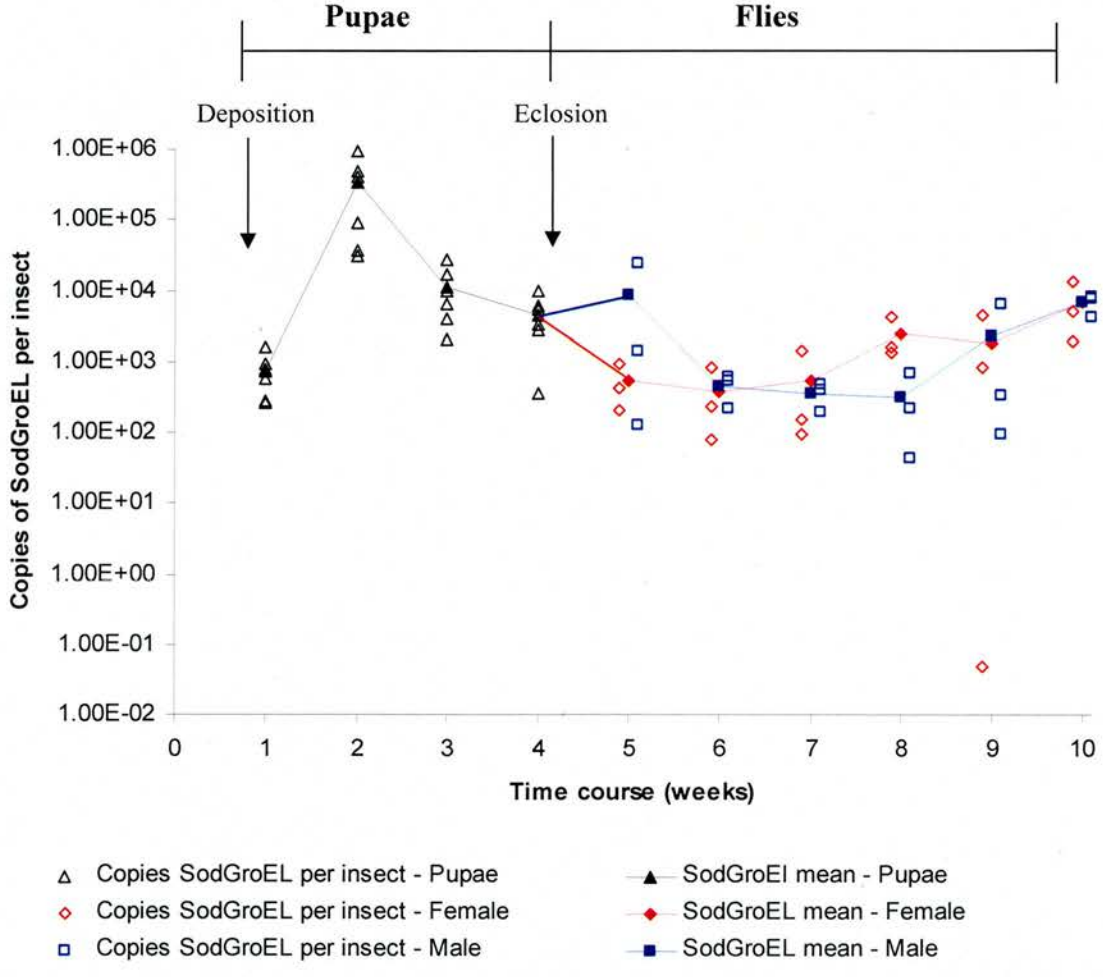
Graph 4.1: The number of copies of *SodGroEL* and pSG1 in tsetse throughout the developmental course, from pupa to adult fly per insect. Data are normalised by insect.

The number of copies of *SodGroEL* increased from a mean of $7.37x10^2$ to a mean of $3.32x10^5$ copies per insect during the first week of pupation. There was a significant increase in the number of copies of *SodGroEL* per insect from week 1 to week 2 (Mann-Whitney *U*, p = 0.005) and a significant decrease from week 2 to week 3 (Mann-Whitney *U*, p = 0.005). The number of copies of *SodGroEL* appears to gradually increase throughout the lifetime of the adult fly, however, there were no significant increases or decreases from week to week throughout this period, with the exception of weeks 9 to 10 during which time there was a significant increase in the copy number of *SodGroEL* (Mann-Whitney *U*, p = 0.045). Overall, throughout the period from eclosion to the end of the study there was no significant change in the number of copies of *SodGroEL* (Mann-Whitney *U*, p = 0.066).

There was a similarly significant increase and decrease in the number of copies of pSG1 between weeks 1 and 2 (Mann-Whitney *U*, p = 0.005), and 2 and 3 (Mann-Whitney *U*, p = 0.005) respectively. The apparent peak in copies of pSG1 at week 9 was shown not to be a significant increase from week 8 (Mann-Whitney *U*, p = 0.471). Despite the high mean number of copies of pSG1 at week 9 ($9.55x10^5$), the range of results at this time point was very large ($4.81x10^2 - 5.32x10^6$). There was, however, a significant decrease between weeks 9 and 10 (Mann-Whitney *U*, p = 0.013).

4.2.3.1 Quantitative PCR of *S. glossinidius* in male and female *G. m. morsitans* throughout the life cycle from pupa to adult

After eclosion it was possible to identify the sex of the flies sampled and three males and three females were randomly chosen from the experimental population (Graph 4.2). Host sex did not appear to affect the population of *S. glossinidius* as there was no significant difference between the number of copies of *SodGroEL* per insect in male and female flies at any point between eclosion and the end of the study (Table 4.1).



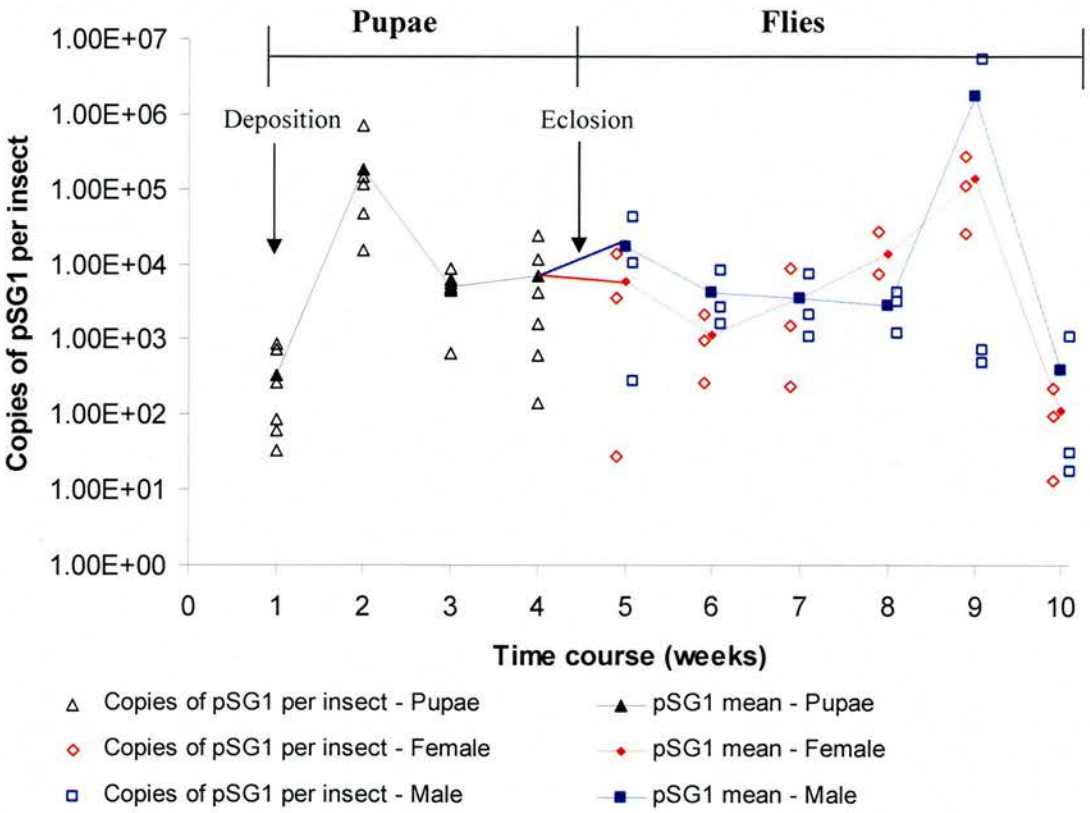
Graph 4.2: The number of copies of *SodGroEL* in tsetse throughout their lifecycle, from pupa to adult fly per insect in male and female flies. Data are normalised by insect.

Week	Mann-Whitney *U* p-value (male/female)
5	0.663
6	1.000
7	0.663
8	0.081
9	1.000
10	1.000

Table 4.1: Mann-Whitney *U* p-values for the null hypothesis that there is no significant difference between the number of copies of *SodGroEL* found in male and female *G. m. morsitans*.

4.2.3.2 Quantitative PCR of pSG1 in male and female *G. m. morsitans* throughout the life cycle from pupa to adult

The number of copies of pSG1 was also reanalysed in order to see if there were any differences between male and female flies (Graph 4.3). Host sex did not appear to affect the copy numbers of pSG1 as there was no significant difference between the number of copies of pSG1 per insect in male and female flies at any point between eclosion and the end of the study (Table 4.2).



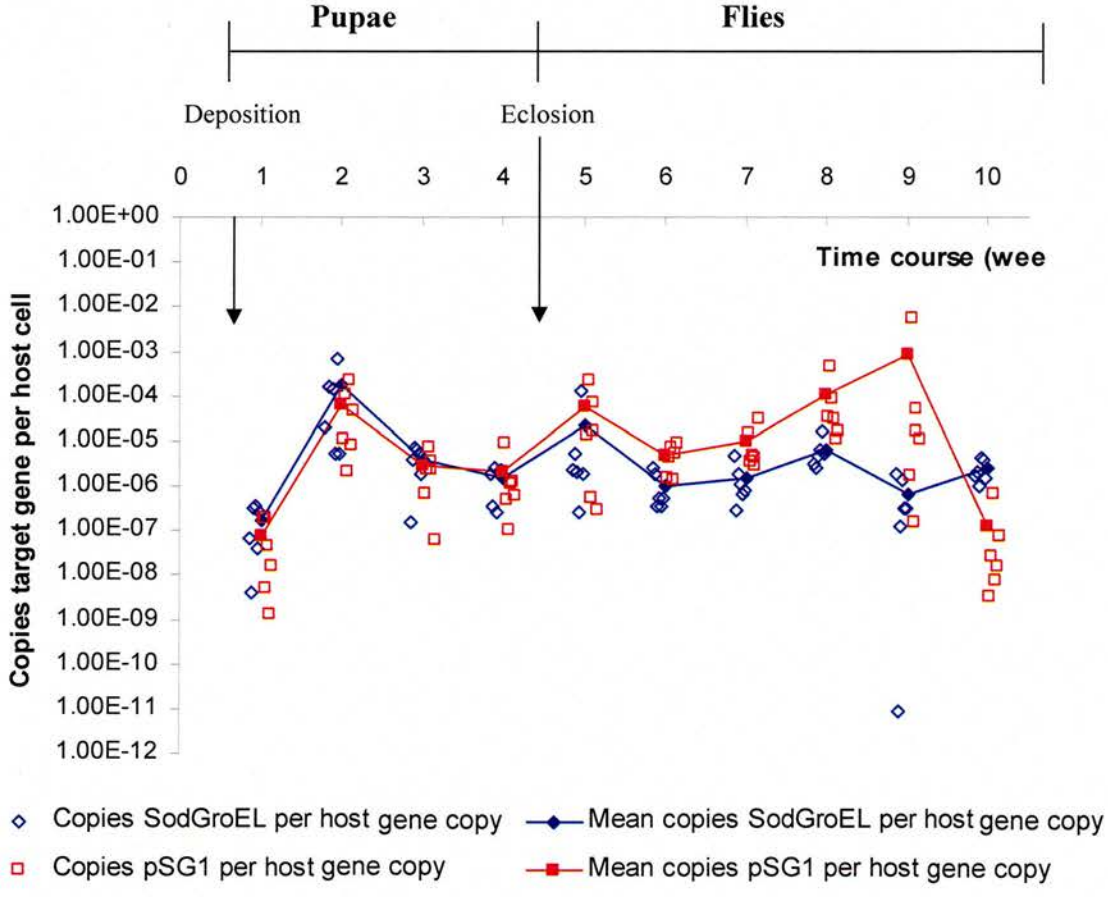
Graph 4.3: The number of copies of pSG1 in tsetse throughout their lifecycle, from pupa to adult fly per insect in male and female flies. Data are normalised by insect.

Week	Mann-Whitney *U* p-value (male/female)
5	0.663
6	0.190
7	1.000
8	0.081
9	0.663
10	1.000

Table 4.2: Mann-Whitney *U* p-values for the null hypothesis that there is no significant difference between the number of copies of pSG1 found in male and female *G. m. morsitans*.

4.2.4 Quantitative PCR of *SodGroEL* and pSG1 density per host cell in *G. m. morsitans* throughout the life cycle from pupa to adult

In order to give a greater depth of information about the dynamics of the *S. glossinidius* population, the density of *SodGroEL* copies per host gene copy (measured by the single copy gene, α-elongation factor) was established (Graph 4.4).



Graph 4.4: Density of *S. glossinidius* and pSG1 relative to host gene copy number. Data are normalised by insect.

The data were statistically analysed using Mann-Whitney *U* tests to see if there were significant changes in the mean number of gene copies or density per host gene copy between adjacent weeks of the time course study (Table 4.3).

Time point comparison	Mann-Whitney *U* p-values for *S. glossinidius* number	Mann-Whitney *U* p-values for *S. glossinidius* density	Mann-Whitney *U* p-values for pSG1 number	Mann-Whitney *U* p-values for pSG1 density
Week1 – Week 2	0.005	0.005	0.005	0.005
Week 2 – Week 3	0.005	0.031	0.005	0.031
Week 3 – Week 4	0.230	0.230	0.575	0.471
Week 4 – Week 5	0.174	0.521	0.936	0.174
Week 5 – Week 6	0.471	0.174	0.379	0.379
Week 6 – Week 7	0.689	0.575	1.000	0.575
Week 7 – Week 8	0.298	0.020	0.337	0.031
Week 8 – Week 9	1.000	0.005	0.471	0.298
Week 9 – Week 10	0.045	0.031	0.013	0.008

Table 4.3: Mann-Whitney *U* p-values for the null hypothesis that there is no significant difference between the number of copies of *SodGroEL* or pSG1 respectively, found between adjacent weeks in *G. m. morsitans*. Significant differences are shown in red.

Although there were significant changes in the mean density of *S. glossinidius* per host gene copy between weeks 7 and 8 (Mann-Whitney *U*, p = 0.020) and weeks 8 and 9 (Mann-Whitney *U*, p = 0.005) there were no significant difference found in the mean number of copies of *SodGroEL* over these time points (Mann-Whitney *U*, p = 0.298 and p = 1.000 respectively). There was also a significant increase in the mean pSG1 density per host gene copy between weeks 7 and 8 (Mann-Whitney *U*, p = 0.031) where no significant increase was seen in the measurement of mean copy numbers of this plasmid (Mann-Whitney *U*, p = 0.337).

Throughout weeks 1 to 5 of the time course study there were no significant differences found between the mean density of *SodGroEL* per host gene copy and the mean density of pSG1 per host gene copy (Table 4.4). From week 6 to the end of the

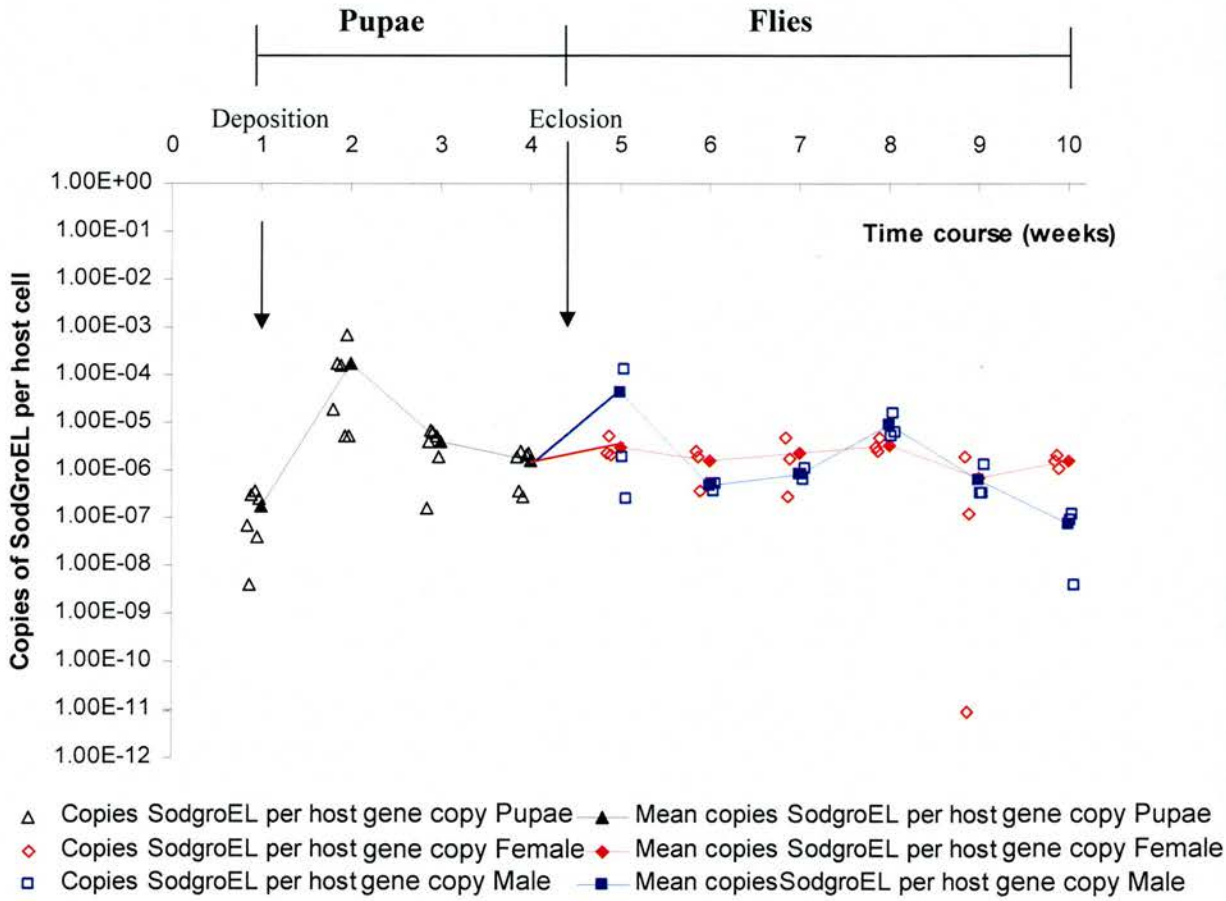
study, there was a significant difference found between the mean density of *SodGroEL* per host gene copy and the mean density of pSG1 per host gene copy, with the exception of week 9 where the difference approached significance (Mann-Whitney *U*, p = 0.055).

Time point comparison	Mann-Whitney *U* p-values for *S. glossinidius* per host gene copy – pSG1 per host gene copy	Significant change
Week 1	0.230	-
Week 2	0.689	-
Week 3	0.471	-
Week 4	0.575	-
Week 5	0.471	-
Week 6	0.031	Increase
Week 7	0.031	Increase
Week 8	0.008	Increase
Week 9	0.055	-
Week 10	0.005	Decrease

Table 4.4: Mann-Whitney *U* p-values for the null hypothesis that there is no significant difference between the density of copies of *SodGroEL* and pSG1 in *G. m. morsitans*.

4.2.4.1 Quantitative PCR of *SodGroEL* density per host cell in male and female *G. m. morsitans* throughout the life cycle from pupa to adult

The number of copies of *SodGroEL* per host gene copy was reanalysed in order to see if there were any differences between male and female flies (Graph 4.5). There was found to be no significant effect of host sex on the density of *SodGroEL* per host gene copy (Table 4.5).



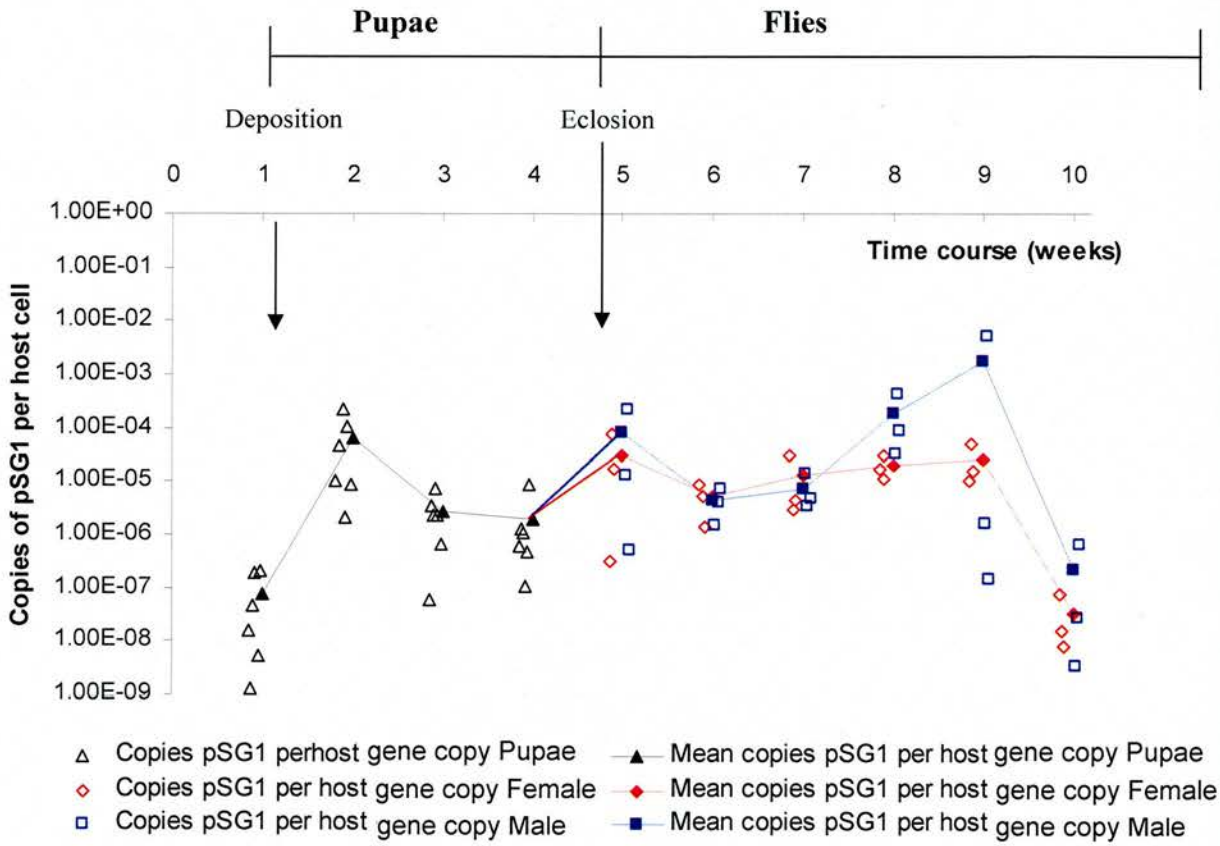
Graph 4.5: Density of *S. glossinidius* relative to host gene copy number in male and female flies. Data are normalised by insect.

Week	Mann-Whitney *U* p-value (male/female)
5	0.663
6	0.663
7	0.663
8	0.081
9	0.663
10	0.383

Table 4.5: Mann-Whitney *U* p-values for the null hypothesis that there is no significant difference between the ratio of copies of *SodGroEL* per host gene copy found in male and female *G. m. morsitans*.

4.2.4.2 Quantitative PCR of pSG1 density per host cell in male and female *G. m. morsitans* throughout the life cycle from pupa to adult

The number of copies of pSG1 per host cell was also reanalysed in order to see if there were any differences between male and female flies (Graph 4.6). There was found to be no effect of host sex on the density of pSG1 per host cell (Table 4.6).



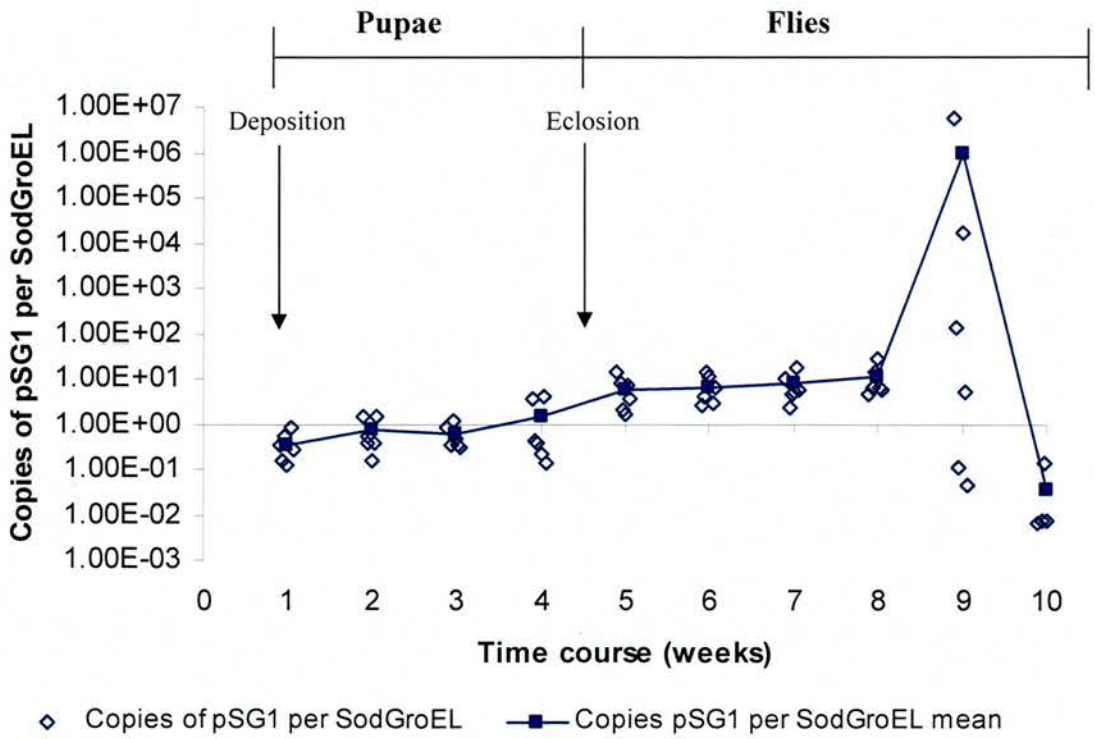
Graph 4.6: Density of pSG1 relative to host gene copy number in male and female flies. Data are normalised by insect.

Week	Mann-Whitney *U* p-value (male/female)
5	1.000
6	1.000
7	1.000
8	0.081
9	0.663
10	1.000

Table 4.6: Mann-Whitney *U* p-values for the null hypothesis that there is no significant difference between the ratio of copies of pSG1 per host gene copy found in male and female *G. m. morsitans*.

4.2.5 Copies of pSG1 per *SodGroEL*

The number of copies of pSG1 per *SodGroEL* was calculated through the developmental course of tsetse (Graph 4.7). There was an apparent increase in the number of copies of pSG1 per *SodGroEL* between weeks 8 and 9, corresponding to the adult fly in the time course study (Table 4.7). However, this peak was not significant (Mann-Whitney *U*, p = 0.810) as the range of copies of pSG1 per *SodGroEL* at this point is so broad. The overall increase in the number of copies of pSG1 per *SodGroEL* between weeks 1 and 9 approaches significance (Mann-Whitney *U*, p = 0.054). The decrease seen in the number of copies of pSG1 per *SodGroEL* between weeks 9 and 10 is significant (Mann-Whitney *U*, p = 0.008), with the mean number of copies dropping from approximately 10^{6} to 10^{-2}.



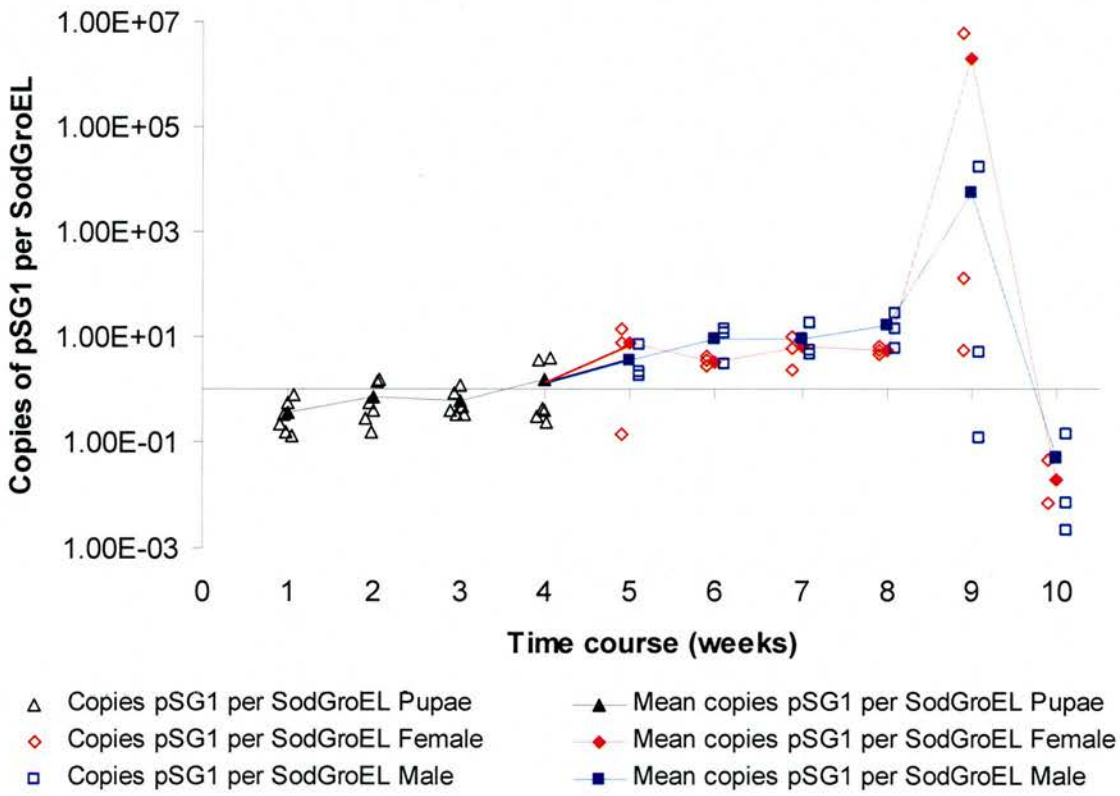
Graph 4.7: Copies of pSG1 per *SodGroEL*. Data normalised by insect.

Time point comparison	Mann-Whitney *U* p-values for pSG1 per *SodGroEL*	Significant change
Week1 – Week 2	0.230	-
Week 2 – Week 3	0.936	-
Week 3 – Week 4	1.000	-
Week 4 – Week 5	0.230	-
Week 5 – Week 6	0.575	-
Week 6 – Week 7	0.575	-
Week 7 – Week 8	0.471	-
Week 8 – Week 9	0.810	-
Week 9 – Week 10	0.008	Decrease

Table 4.7: Mann-Whitney *U* p-values for the null hypothesis that there is no significant difference between the number of copies of pSG1 per *SodGroEL* found between adjacent weeks in *G. m. morsitans*.

4.2.5.1 Copies of pSG1 per *SodGroEL* in male and female flies

The number of copies of pSG1 per *SodGroEL* was reanalysed to see if there were any differences between male and female flies (Graph 4.8). There was no significant effect of host sex on the copy number of pSG1 per *SodGroEL* (Table 4.8).



Graph 4.8: Copies of pSG1 per *SodGroEL* in male and female flies. Data normalised by insect.

Week	Mann-Whitney *U* p-values for pSG1 per *SodGroEL* female / male
Week 5	0.663
Week 6	0.383
Week 7	1.000
Week 8	0.190
Week 9	0.383
Week 10	1.000

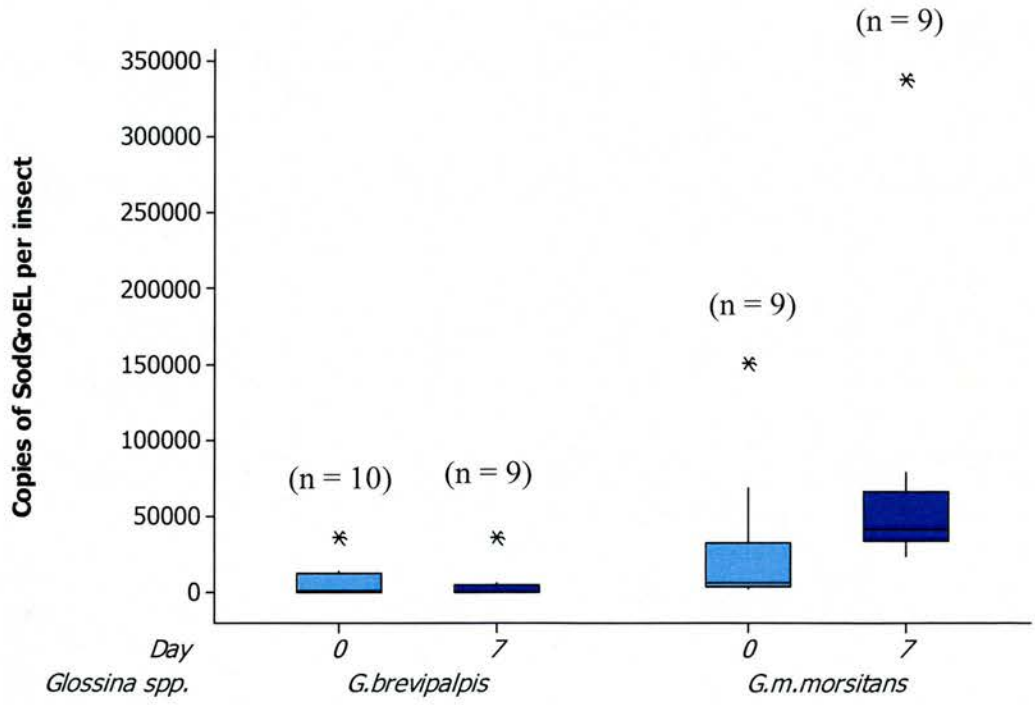
Table 4.8: Mann-Whitney *U* p-values for the null hypothesis that there is no significant difference between the ratio of copies of pSG1 per *SodGroEL* found in male and female *G. m. morsitans*.

4.2.6 RNA extraction from time course samples

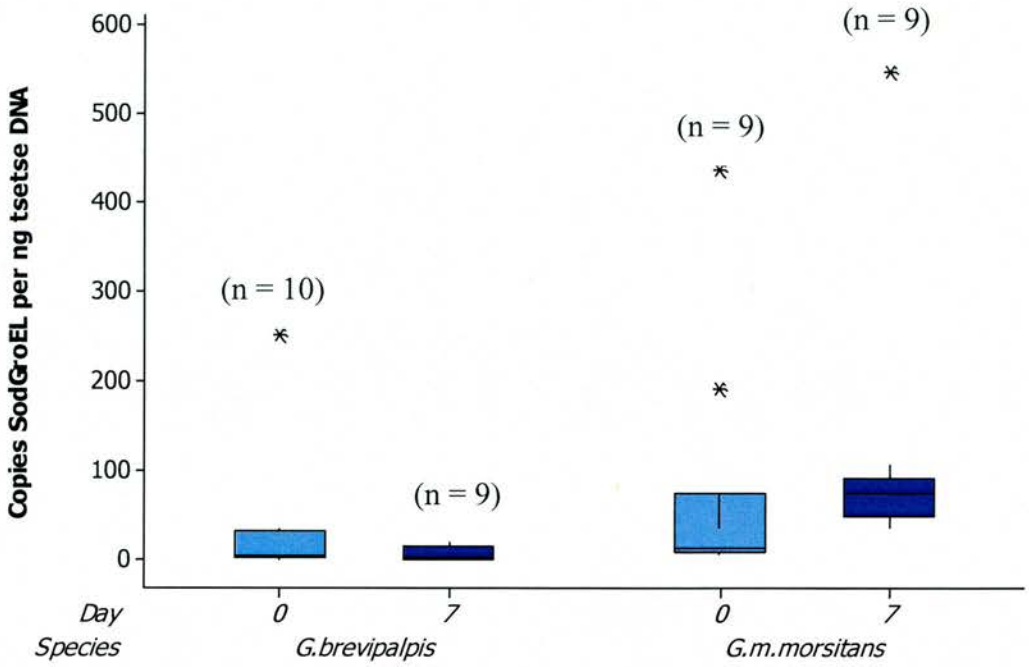
RNA was extracted from the samples over the time course of the tsetse fly life cycle simultaneously with DNA. This technique yielded very poor results for RNA and it proved impossible to clean the samples of contaminating DNA sufficiently to enable qPCR. Therefore, it was not possible to carry out the intended studies of *S. glossinidius* gene expression over the life cycle of the fly.

4.2.7 Quantitative PCR for *S. glossinidius* in 0 and 7 day old *G. brevipalpis* and *G. m. morsitans* pupae

The number of copies of *SodGroEL* was analysed in newly deposited and seven day old *G. brevipalpis* and *G. m. morsitans* pupae in order to see if there were differences in the *S. glossinidius* population sizes and dynamics of these two species of tsetse (Graph 4.9). The data was also normalised by the total DNA extracted from each individual and expressed as gene copies per ng DNA in order to compare the bacterial populations relative to the insects size (Graph 4.10). These two species of *Glossina* are morphologically distinct, with *G. brevipalpis* being larger than *G. m. morsitans* (Figure 4.3).



Graph 4.9: Number of copies of *SodGroEL* per insect in 0 and 7 day post-deposition *G. brevipalpis* and *G. m. morsitans* pupae. Outlying data points are denoted with an asterisk.



Graph 4.10: Number of copies of *SodGroEL* per ng tsetse DNA in 0 and 7 day post-deposition *G. brevipalpis* and *G. m. morsitans* pupae. Outlying data points are denoted with an asterisk.

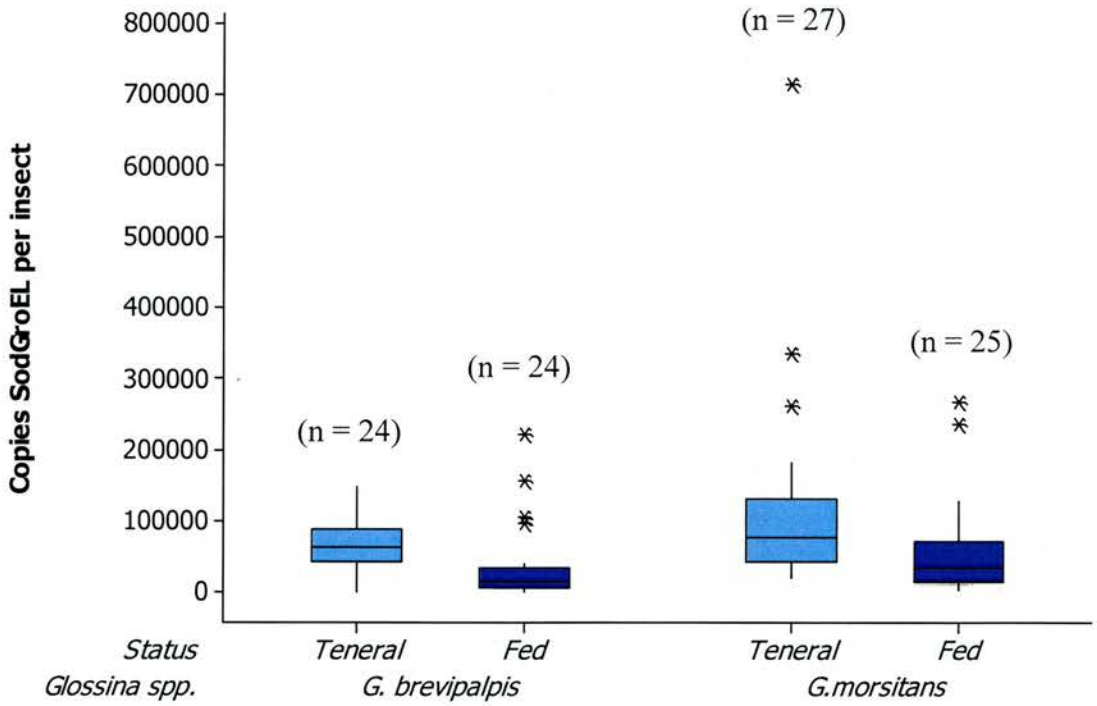
Figure 4.3: *G. brevipalpis* (top) and *G. m. morsitans* (bottom) to show the difference in size of the two species.

G. brevipalpis pupae were found to have a mean of $7.12x10^3$ copies of *SodGroEL* per insect in pupae on the day of deposition. This was not significantly different from the mean number of copies, $2.76x10^4$ found in *G. m. morsitans* pupae at day 0 (Mann-Whitney *U*, $p = 0.066$). Seven days later, the difference in mean *SodGroEL* copy numbers had increased between the two species of *Glossina* to become significant (Mann-Whitney *U*, $p = 0.001$). The number in *G. brevipalpis* pupae fell to $5.24x10^3$, although the decrease from day 0 was not significant (Mann-Whitney *U*, $p = 0.427$). At the same time the mean copy number of *SodGroEL* in *G. m. morsitans* pupae increased significantly (Mann-Whitney *U*, $p = 0.016$) to $7.6x10^4$.

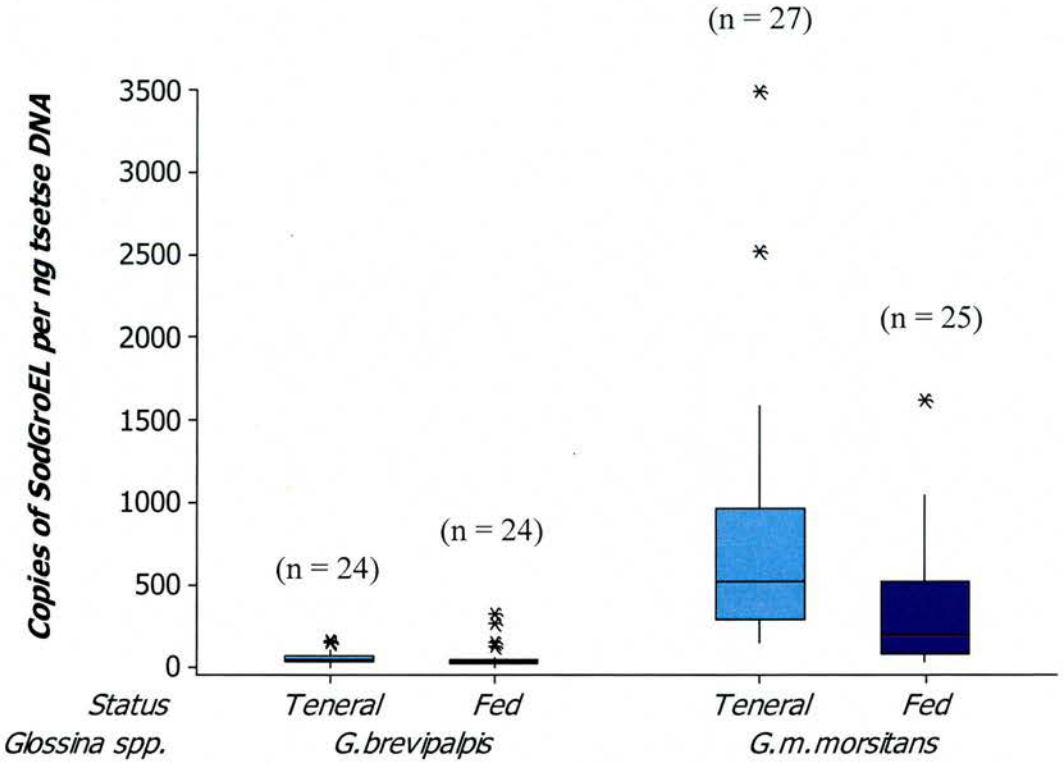
When the data was represented as copies of *SodGroEL* per ng DNA the pattern of the data remained constant, with *G. brevipalpis* showing no significant increase or decrease in copy numbers (Mann-Whitney *U*, $p = 0.307$) whilst they significantly increased in *G. m. morsitans* (Mann-Whitney *U*, $p = 0.025$). The differences between the two species also remained equivalent, with no difference seen in copy number at day 0 (Mann-Whitney *U*, $p = 0.111$) and a significantly higher number of copies of *SodGroEL* in *G. m. morsitans* at day 7 (Mann-Whitney *U*, $p = 0.004$) reflective of the *S. glossinidius* population increase in only *G. m. morsitans*.

4.2.8 Quantitative PCR for *S. glossinidius* in teneral and 10 day old *G. m. morsitans* and *G. brevipalpis* flies

The number of copies of *SodGroEL* was analysed in teneral and fed *G. brevipalpis* and *G. m. morsitans* flies in order to see if there were differences between the *S. glossinidius* population sizes and dynamics of these two species of tsetse (Graph 4.11). Once again the data was normalised by the total DNA extracted from each individual and expressed as gene copies per ng DNA in order to compare the bacterial populations relative to the insects size (Graph 4.12).



Graph 4.11: Number of copies of *SodGroEL* per insect in teneral and 10 day old fed *G. brevipalpis* and *G. m. morsitans* flies. Outlying data points are denoted with an asterisk.



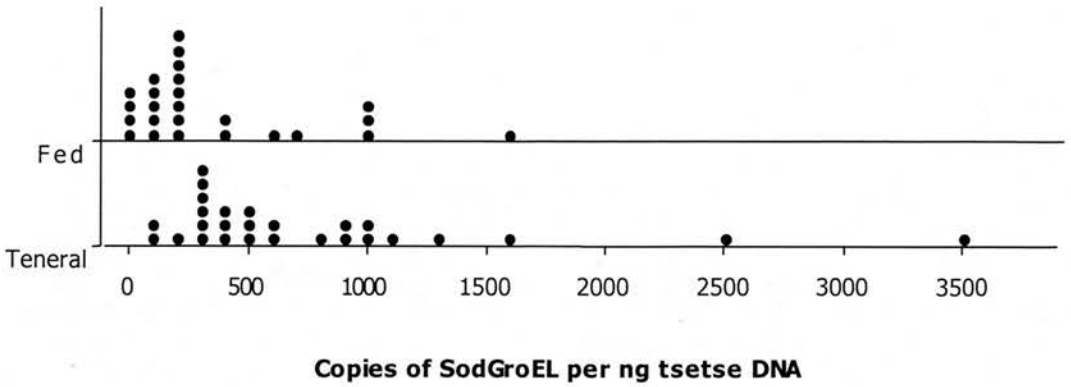
Graph 4.12: Number of copies of *SodGroEL* per ng tsetse DNA in teneral and 10 day old fed *G. brevipalpis* and *G. m. morsitans* flies. Outlying data points are denoted with an asterisk.

Teneral *G. brevipalpis* were found to have a mean of 6.52×10^4 copies of *SodGroEL* per insect. This decreased significantly to a mean of 3.61×10^4 in ten day old fed flies (Mann-Whitney *U*, p = 0.001). A similarly significant decrease was observed in *G. m. morsitans*, from a mean of 1.15×10^5 to 5.73×10^4 copies of *SodGroEL* per insect in teneral and fed flies respectively (Mann-Whitney *U*, p = 0.003). The number of copies of *SodGroEL* in *G. m. morsitans* was higher than in *G. brevipalpis* at both time points, despite the larger body-size of the latter species. Whilst this difference was not found to be significant in teneral flies (Mann-Whitney *U*, p = 0.286), the number of copies of *SodGroEL* in fed flies in *G. m. morsitans* was significantly higher than in *G. brevipalpis* (Mann-Whitney *U*, p = 0.040).

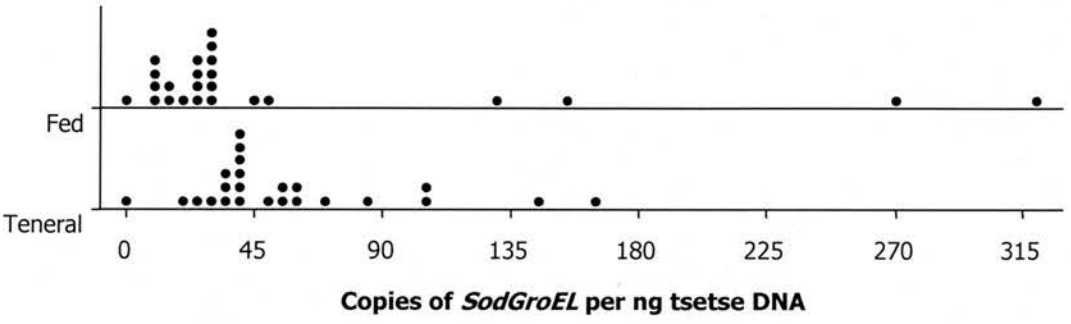
Both *G. m. morsitans* (Mann-Whitney *U*, p = 0.002) and *G. brevipalpis* (Mann-Whitney *U*, p = 0.008) were found to have significantly lower copy numbers of *SodGroEL* per ng DNA in fed flies than in teneral flies (Graph 4.12). Teneral *G. m. morsitans* flies had significantly higher copy numbers of *SodGroEL* per ng DNA when compared to teneral *G. brevipalpis* flies (Mann-Whitney *U*, p <0.001), however there was no significant difference between the two species after feeding (Mann-Whitney *U*, p = 0.122).

The number of copies of *SodGroEL* per ng DNA was used to compare the population of *S. glossinidius* between *G. m. morsitans* and *G. brevipalpis* in order to compensate for the larger size of the latter species (Graph 4.13). Four teneral *G. m. morsitans* were seen to have larger numbers of the gene than the majority of the sample population. These data were found to be significantly aggregated by measuring the index of dispersion (737). This value was highly significant ($\chi^2_{26} = 1.92 \times 10^4$, p <0.001). In fed *G. m. morsitans* the data were also found to be significantly aggregated (Index of dispersion = 26, $\chi^2_{23} = 5.99 \times 10^2$, p <0.1). Similar results were obtained for both teneral (Index of dispersion = 47.5, $\chi^2_{24} = 1.14 \times 10^4$, p <0.001) and fed (Index of dispersion = 120, $\chi^2_{23} = 2.76 \times 10^3$, p <0.001) *G. brevipalpis*.

A:



B:



Graph 4.13: Dot plots of the number of copies of *SodGroEL* in teneral and fed tsetse flies. Graph A shows *G. m. morsitans* and graph B shows *G. brevipalpis*. Notice the difference in the axis numbering.

4.2.9 Quantitative PCR of plasmid copy number in *S. glossinidius* cultures *in vitro*

The number of copies of plasmids associated with *S. glossinidius in vitro* was found to vary greatly between isolates from different species of *Glossina* (Table 4.9). In *S. glossinidius* GA, isolated from *G. austeni*, there were 0.9 copies of pSG1, 0.7 copies of pSG2, 0 copies of pSG3 and 1.8 copies of pSG4 per copy of *SodGroEL*. In GP, isolated from *G. p. palpalis*, there were 19.0 copies of pSG1, 7.8 copies of pSG2, 661.3 copies of pSG3 and 36.5 copies of pSG4 per copy of *SodGroEL*.

Isolate	pSG1	pSG2	pSG3	pSG4
S. glossinidius GA	0.9	0.7	0	1.8
S. glossinidius GP	19	7.8	661.3	36.5
Average	**9.9**	**4.2**	**330.6**	**19.2**
Average per *S. glossinidius cell*	**79.2**	**33.6**	**2644.8**	**153.6**

Table 4.9: Plasmid copy numbers per *SodGroEL* in two isolates of *S. glossinidius*: GA and GP. The average value is the mean of the plasmid copy numbers in the two isolates. The average per *S. glossinidius* cell is the average value multiplied by eight as there are eight copies of *SodGroEL* per *S. glossinidius* cell.

4.2.10 Quantitative PCR of plasmid copy number in lab colony flies, *G. p. palpalis* and *G. austeni*

The copy number of plasmids associated with *S. glossinidius* was found to be very similar in the two species of tsetse that were tested (Table 4.10). In *G. austeni* there was a mean of 0.5 copies of pSG1 (n = 5), 1.5 copies of pSG2 (n = 5), 5.1 copies of pSG3 (n = 5) and 0.5 copies of pSG4 (n = 5) per copy of *SodGroEL*. In *G. p. palpalis* there was a mean of 0.7 copies of pSG1 (n = 5), 1.6 copies of pSG2 (n = 3), 12.4 copies of pSG3 (n = 5) and 1.0 copies of pSG4 (n = 5) per copy of *SodGroEL*.

***Glossina* spp.**	**pSG1**	**pSG2**	**pSG3**	**pSG4**
G. austeni	0.5	1.5	5.1	0.5
G. p. palpalis	0.7	1.6	12.4	1
Average	**0.6**	**1.6**	**8.8**	**0.8**
Average per *S. glossinidius cell*	**4.8**	**12.8**	**70.4**	**6.4**

Table 4.10: Plasmid copy numbers per *SodGroEL* in two species of *Glossina*: *G. austeni* and *G.palpalis*. The average value is the mean of the plasmid copy numbers in the two isolates. The average per *S. glossinidius* cell is the average value multiplied by eight as there are eight copies of *SodGroEL* per *S. glossinidius* cell.

Given the findings of this present work that there are approximately 8 copies of *SodGroEL* per *S. glossinidius* cell, these results may be multiplied appropriately to give an estimate of the actual number of each plasmid per cell. This results in an average for the two species of tsetse of: 4 copies of pSG1, 11 copies of pSG2, 62 copies of pSG3 and 6 copies of pSG4 per *S. glossinidius* cell.

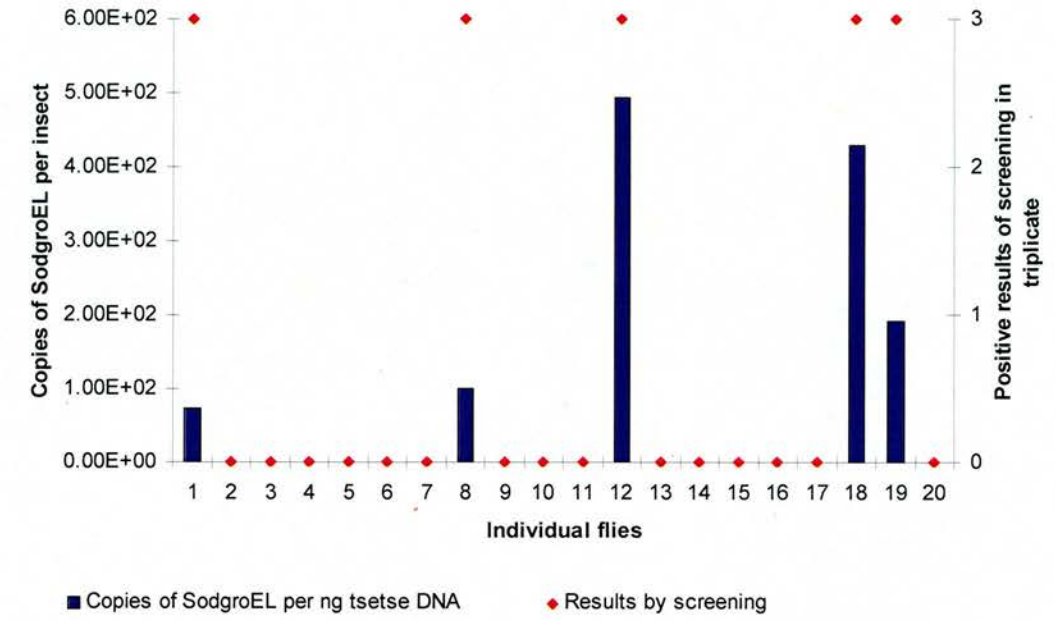
4.2.11 Quantitative PCR of wild *G. pallidipes* and *G. m. morsitans* for *S. glossinidius*

The populations of *S. glossinidius* in wild *G. pallidipes* and *G. m. morsitans* were quantified using qPCR (Graph 4.14). The quantitative analysis of *S. glossinidius* populations was compared to the results of screening for *S. glossinidius* infections by PCR (see Chapter 5).

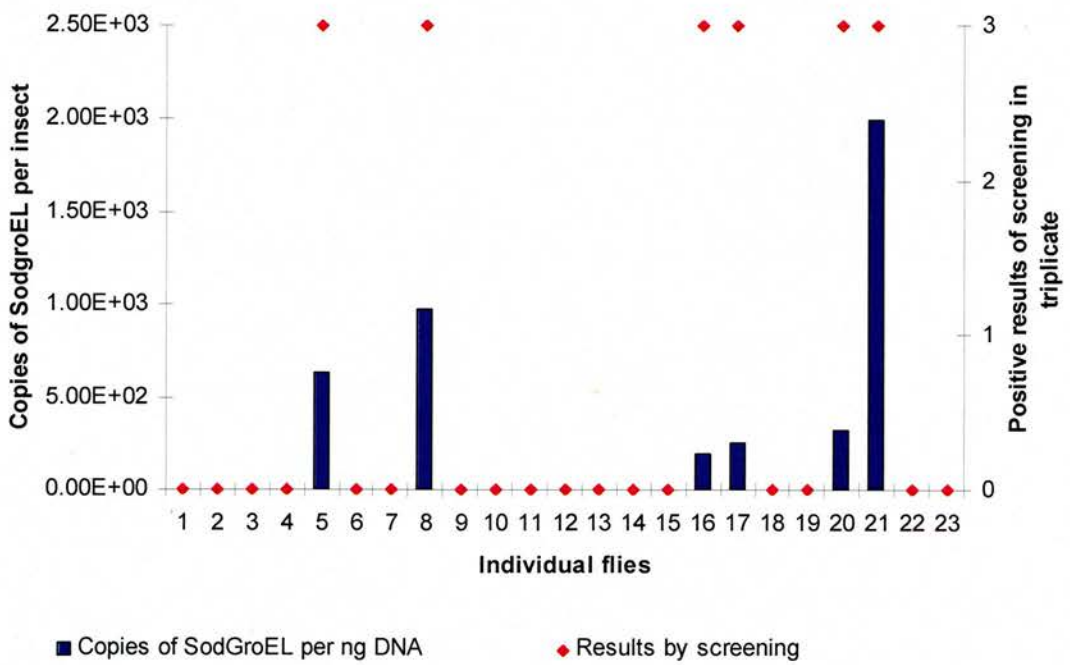
The proportion of flies testing positive for *SodGroEL* was 25% (5/20) for *G. m. morsitans* and 26% (6/23) for *G. pallidipes*. The correlation between the results of qPCR and diagnostic PCR is 100% for both *G. m. morsitans* and *G. pallidipes* showing that the diagnostic PCR used in this work is a reliable indicator of the presence or absence of *S. glossinidius* and is not prone to false positive or false negative results.

The size of *S. glossinidius* populations in the two species of *Glossina* is markedly different. The number of copies of *SodGroEL* quantified per *G. m. morsitans* ranged from 74 to 493 whilst values for *G. pallidipes* ranged from 197 to 2000 copies. The number of copies of *SodGroEL* in wild *G. m. morsitans* was found to be approximately two orders of magnitude lower than in their laboratory colony counterparts (Section 4.2.8).

G. m. morsitans:



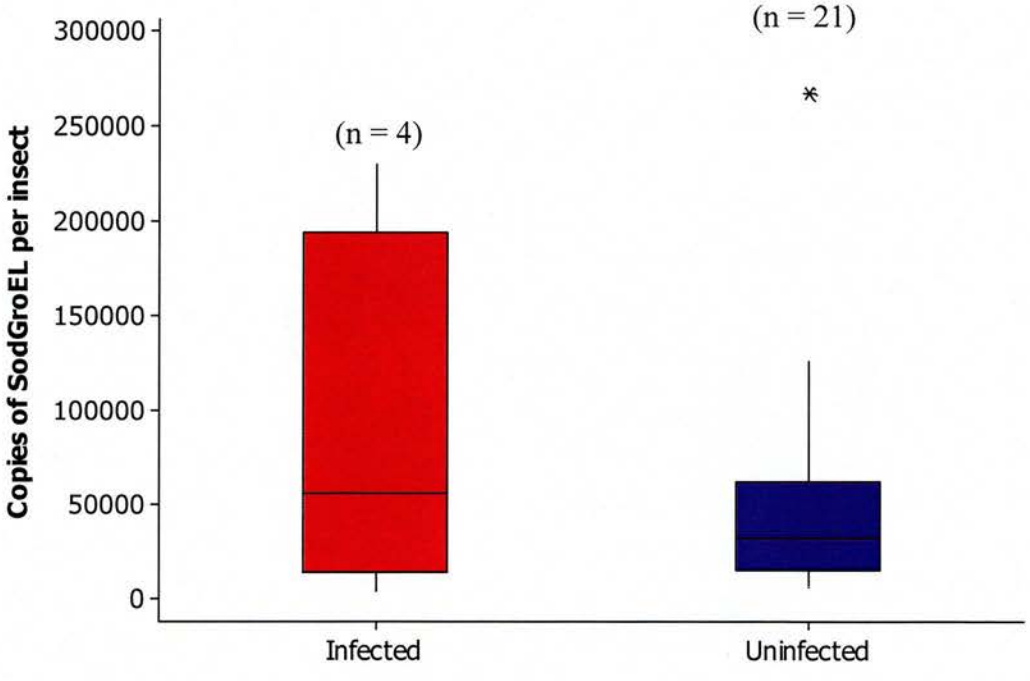
G. pallidipes:



Graph 4.14: The number of copies of *SodGroEL* per insect and the results of diagnostic PCR in both wild *G. m. morsitans* and *G. pallidipes*.

4.2.12 Quantitative PCR of infected and uninfected flies

The size of *S. glossinidius* populations in infected and refractory *G. m. morsitans* were measured at 10 days after the infective blood meal in order to establish if there were any differences between the number of bacteria in the two groups (Graph 4.15). Flies were screened by PCR in order to determine whether they had become infected by *T.b.brucei*. Four of the twenty-five flies sampled (16%) were found to be infected.



Graph 4.15: The number of copies of *SodGroEL* in *G. m. morsitans* that have been challenged with trypanosomes. Data has been normalised by insect. The outlying value in the refractory data set is marked by an asterisk.

There was no significant difference in the number of copies of *SodGroEL* found in infected and refractory *G. m. morsitans* at 10 days post *T.b.brucei* challenge (Mann-Whitney *U*, p = 0.578). This remained the case when the outlying, and possibly anomalous, value in the refractory data set was removed from the analysis (Mann-Whitney *U*, p = 0.462).

4.3 Discussion

S. glossinidius was found to contain approximately eight genome copies per cell. Polyploidy is also exhibited by the endosymbiont of the pea aphid, *Acyrthosiphon pisum, Buchnera aphidicola* which contains several hundred genome copies per bacterial cell (Komaki & Ishikawa, 1999). In *Buchnera* spp. this characteristic is thought to act as a method of gene expression regulation, a measure perhaps necessary due to the extreme genome size reduction and accompanying gene loss undergone by this bacterium (Wolschin *et al.*, 2004). Changes in the genome copy number have been seen in rapidly growing enterobacteria, which may contain several copies per cell (Skarstad *et al.*, 1985). This increase, however, simply results from the initiation of several rounds of chromosome replication per cell cycle during periods of rapid growth, and the ploidy of such bacteria will decrease to one per cell as the growth rate declines (Zyskind & Smith, 1992).

The number of genome copies in *S. glossinidius* cells was measured *in vitro* during exponential growth phase in order to enable the enumeration of bacterial cells using both qPCR and total cell counts. During this phase of growth bacteria are undergoing replication and limiting factors of the growth conditions have not been reached, so the viable to total bacterial cell ratio is at a maximum. It is possible that the multiple genome copies recorded in the present work were a result of rapid reproduction of the bacteria and do not represent the situation during other phases of bacterial growth.

4.3.1 *S. glossinidius* population size and dynamics

The peak in the population size of *S. glossinidius*, observed in pupae one week after deposition, is roughly consistent with the doubling time of the bacterium (26 hours) as established in Chapter 3 and in Matthew *et al.* (Matthew *et al.*, 2005). Over the course of seven days, the *S. glossinidius* population was seen to increase (by approximately 2^9) from a mean of 7.37×10^2 copies of *SodGroEL* per insect to a mean of 3.32×10^5 copies. This corresponds with approximately nine generations of bacterial replication and results in an approximate generation time of 19 hours. The generation time of *S. glossinidius* found *in vitro* was approximately 26 hours (Matthew *et al.*, 2005). The disparity between these two values is probably due to the sub-optimal growth conditions *in vitro*.

Given the multiple genome copies that have been found in *S. glossinidius* cells by this study, it is possible that increases seen in the copy number of *SodGroEL* represent further genome multiplication within the cell rather than solely indicating an increase in bacterial cell numbers. Further work to examine the genome complement of *S. glossinidius* cells at different stages of growth and under different conditions, such as temperature or nutrient-limited growth medium, would help to clarify this point

The significant increase in the *S. glossinidius* population in early pupation is apparent when either population size or population density is measured. However, no such peak is recognised in the recent work evaluating the dynamics of the bacterial symbionts of *Glossina* spp. by Rio *et al.* (Rio *et al.*, 2005). This may be due to the choice of sampling time points at which *S. glossinidius* population density was measured in that study. Measurements taken at deposition and day 15 of pupation would fall on either side of the described peak of growth, meaning that it would pass unnoticed.

The quantification of the density of *S. glossinidius* in tsetse over their developmental course in this work resulted in an estimation 2 – 3 orders of magnitude smaller than that arrived at in the study by Rio *et al.* (Rio *et al.*, 2005). In addition the range of *S. glossinidius* densities per host gene copy over the study period were found to cover a broad range (c. 10^{-5} to c. 10^{-7}), whilst Rio *et al.* measured a much smaller range of values. The disparities in the results of these two studies may be due to a difference in the tsetse colony used. The twofold increase in *S. glossinidius* density per host gene copy, between teneral and two week old adult flies, that was found by Rio *et al.*, was not mirrored in this study in either the bacterial density measured nor the bacterial population size in the fly as a whole. Although the sample size in the study of the developmental course was smaller in this work, further experiments to examine the difference between teneral and fed flies upheld the conclusion that, in *G. m. morsitans*, there was no increase in *S. glossinidius* population size or density at this stage of development and there was in fact a significant decrease from teneral to fed flies. This finding was also the case in *G. brevipalpis* flies.

At each time point sampled, both the bacterial population size and density were seen to be fairly consistent between individual flies with no difference found between male or female tsetse. This suggests that there is some level of regulation of the symbiont population by the tsetse. It would seem, however, that the rapid increase in the copies of *SodGroEL* measured in the first week after pupae deposition is indicative of an absence of effective regulation at this stage. This is possibly due to the physiological characteristics of the pupae at this stage of development – as a fairly homogenous mass of cells with little apparent organisation (Figure 1).

The absence of any significant difference between the population size or density of *S. glossinidius* in male and female flies was unexpected. As *S. glossinidius* is thought to be exclusively vertically transmitted, the male host represents a 'dead end' for the symbiont as there is no potential for its transmission. Studies of *Drosophila* spp. have found females to maintain high populations of *Wolbachia* spp. throughout their

life whilst males gradually lose their infective status with age (Hoffmann *et al.*, 1986). The fact that this is not the case for *S. glossinidius* may suggest that some horizontal transmission occurs, allowing male flies a route to pass on their symbiont to other tsetse.

4.3.2 Methods of normalisation of qPCR data

The majority of the qPCR data in this chapter were expressed as "copies per insect". In this way the bacterial population size present in the individual insects was represented for comparison between time points. The DNA concentration of each sample was measured and compared within sample groups to ensure that the variation between samples was not disproportionate. It was chosen to present the data as gene copies per insect to give a clearer picture of the *in vivo* dynamics of the *S. glossinidius* population. Where the comparison of two different *Glossina* species was necessary, both the gene copies per insect and the gene copies per ng DNA were analysed. This was done in order to examine both the size of the *S. glossinidius* population and the number of bacteria relative to the size of the insect, as *G. brevipalpis* is a significantly larger insect than *G. m. morsitans*.

Other studies, such as the recent work by Rio (Rio *et al.*, 2005), have chosen to look at the density, rather than the population size of the symbiont by normalising the data by the number of host gene copies present in the sample. The results obtained by this approach are subject to the differing developmental rates of host and symbiont and this factor may lead to a disparity between changes in population size and density measurements. This effect is discussed by Sakurai (Sakurai *et al.*, 2005) in relation to two symbionts of the pea aphid, *A.pisum*. In that study, population size measurements showed "simple growth and plateau dynamics" whereas density

measurements showed a transient drop and peak, where the aphid development sped and slowed relative to the bacterial growth.

At week nine of the time course study, corresponding to a four week old adult fly, there was a significant drop in the density of *S. glossinidius* per host gene copy. This was not, however, echoed in the population size of *S. glossinidius*.

The number of copies of *SodGroEL* per ng DNA was compared in *G. brevipalpis* and *G. m. morsitans*. *G. brevipalpis* was found to have consistently significantly lower copy numbers of *SodGroEL* than *G. m. morsitans*. In their semi-quantitative study of *S. glossinidius* population, Cheng and Aksoy (1999) also concluded that *G. brevipalpis* had significantly lower levels of this symbiont than *G. m. morsitans* and *G. p. palpalis*. Moloo and Shaw (1989) also observed low symbiont levels in *G. brevipalpis* by microscopy.

The diverse levels of *S. glossinidius* infection in various species of tsetse may reflect differences in the respective strains of *S. glossinidius* or different regulation by the immune system of the host. *G. brevipalpis* is a member of the *fusca* group, the ancestral subgenus of *Glossina* spp. and the most divergent group (Chen *et al.*, 1999). By contrast the other three subgenera, *morsitans*, *palpalis* and *austeni* are more closely related. Cheng and Aksoy (1999) found that the introduction of high levels of *S. glossinidius* from *G. m. morsitans* to *G. brevipalpis* was pathogenic. Since strains of *S. glossinidius* from different *Glossina* spp. have been shown to have so few differences, by analysis of their 16S rDNA, it is possible that differences in host-symbiont relationships of different species of tsetse and their strains of *S. glossinidius* are due to host-mediated factors.

The relatively broad spectrum of the number of copies of *SodGroEL* found in teneral flies in the time course data set was also seen when a larger group of teneral *G. m.*

morsitans were studied. This was not the case, however, when the number of copies of *SodGroEL* was quantified in *G. brevipalpis*. There was a broader spectrum of *SodGroEL* copy numbers found in fed *G. brevipalpis* than in teneral flies. This is a reversal of the situation in *G. m. morsitans*.

A significant increase in the population size of *S. glossinidius* was seen in *G. m. morsitans* pupae sampled at 0 and 7 days post-deposition. This showed a similar trend to the results of *S. glossinidius* population size measurements through the developmental course of the tsetse, though to a smaller extent.

The differences between the results obtained in different experiments may be explained by the different methods of DNA extraction used. The study of the entire developmental course, which showed a 450 fold increase in *SodGroEL* between weeks 1 and 2, used a simultaneous DNA and RNA extraction technique. The comparative study between *G. brevipalpis* and *G. m. morsitans* used only a DNA extraction and showed an increase of 2 fold of *SodGroEL* in *G. m. morsitans* between 0 and 7 days post-deposition. Whilst the former method of DNA extraction was simple and efficient, the latter method was more complicated and necessitated a long period of optimisation before satisfactory results could be achieved. In addition the number of copies of *SodGroEL* was approximately two orders of magnitude greater in the comparative experiment than in the study of the developmental course.

The greater efficiency of the DNA extraction used for the comparison of *SodGroEL* copy numbers in *G. m. morsitans* and *G. brevipalpis* may account for the increased number of *S. glossinidius* present in the sample compared to DNA collected in the study of the developmental course. However, this does not explain the extreme peak in *SodGroEL* copy numbers seen at week 2 of the latter experiment, which exceeds the number found in the comparative experiment.

When the population of *S. glossinidius* was investigated in wild *G. m. morsitans* and *G. pallidipes* approximately 25% of the flies were found to be infected by the bacterium. These findings tallied exactly with the results of the screening study on the same samples, suggesting that the protocol used to screen large numbers of wild flies for *S. glossinidius* infection is robust and not prone to false positives or negatives (Chapter 5).

In addition, the number of copies of *SodGroEL* was 1 to 3 orders of magnitude smaller in wild *G. m. morsitans* and *G. pallidipes* populations than in the laboratory colony flies examined. This may be a result of the greater environmental stresses experienced by the host in the wild compared to under laboratory conditions that could influence the resources available to *S. glossinidius* as well as the regulation of this symbiont.

4.3.3 Trypanosome data

There are many factors that may affect the susceptibility of tsetse flies to infection by trypanosomes. In the wild these include endogenous (vector), environmental, parasite and mammalian host factors, all of which influence the infection rates of tsetse populations. Under laboratory conditions, many of these variables are controlled, for instance the age at infective feed and the number of parasites present in that feed (Jordan, 1974; Molyneux, 1980). Apart from these factors, trypanosome infection rates of tsetse differ according to the species of *Glossina*, the species of *Trypanosoma* and between different populations of flies.

Previous work has shown that *G. brevipalpis* has a low susceptibility to infection by trypanosomes (Moloo *et al.*, 1987; Moloo & Kutuza, 1988a, b). *G. brevipalpis* from the laboratory colony held at the Centre for Tropical Veterinary Medicine, University

of Edinburgh have been found to have a far lower rate of infection (4%) with *T.b.brucei* than *G. m. morsitans* (20%) using laboratory colony flies (Personal communication, E. MacLeod). Considering the hypothesis that high levels of *S. glossinidius* contribute to increased susceptibility of *Glossina* spp. to trypanosome infection, the low levels found in *G. brevipalpis* correspond with the low rates of trypanosome infection.

Previous studies have suggested that there is a link between the susceptibility of *Glossina* spp. to the establishment of trypanosome infections and the presence of the endosymbiont *S. glossinidius* in the midgut epithelial cells (Welburn & Maudlin, 1991) (Maudlin & Ellis, 1985) (Welburn *et al.*, 1993). The detection of *S. glossinidius* in both infected and uninfected tsetse in studies by Moloo and Shaw (1989) and Geiger *et al.* (2005) led these authors to question the "permissive role" of *S. glossinidius* in the establishment of trypanosome infections (Geiger *et al.*, 2005). However, Welburn and Maudlin (1991) established that there was a quantitative relationship between *S. glossinidius* infection and tsetse susceptibility, so the mere presence or absence of the symbiont is not necessarily conclusive and the quantification of the bacterial population in the fly is needed.

In the current work the population size of *S. glossinidius* in *G. m. morsitans* with midgut trypanosome infections was found to be no different to the *S. glossinidius* population size in *G. m. morsitans* which were refractory to trypanosome infection. This finding is in accordance with a similar study by Rio *et al.* (Rio *et al.*, 2005). The similarity of *S. glossinidius* population sizes in susceptible and refractory flies found here does not necessarily indicate that the symbiont level has no effect on the course of infection of tsetse with trypanosomes as bacterial populations are dynamic and the point at which the relevant interaction would occur is whilst the fly is in the teneral state.

It is well established that teneral tsetse are more susceptible to trypanosome infection than fed flies (Welburn & Maudlin, 1992; Welburn *et al.*, 1994). Therefore, any potential effect of symbiont population size on the refractoriness of the fly would be negated after the first feed. Ideally the symbiont levels of individual tsetse would be measured before the first feed and once again after the fly had been challenged with trypanosomes. Currently, however, DNA extraction techniques for the quantification of symbionts involve the homogenisation of the entire fly and subsequent feeding experiments on that individual are clearly impossible.

4.3.4 Plasmid copy numbers

4.3.4.1 pSG1 copy numbers

The ratio of pSG1 copies per *S. glossinidius* genome were found to stay constant between approximately 1:1 and 10:1 for most of the developmental course of the tsetse fly. The one exception to this rule was seen at week ten of the study, corresponding to a five week old adult fly, where the ratio dropped to below one copy of pSG1 per *S. glossinidius* genome. The consistent level of pSG1 copy numbers over time suggests that this plasmid is able to persist in the bacterial population and that its copy number in the cell may be controlled. For the persistence of plasmids one of two mechanisms is necessary: either selection for the

genes carried on the plasmid that are beneficial to the host or infectious transmission and maintenance as genetic parasites (Bergstrom *et al.*, 2000).

The plasmid pSG1 is the largest of the four extrachromosomal elements associated with *S. glossinidius* (Darby *et al.*, 2005). Encoded on this plasmid are genes for siderophore biosynthesis and transport (achromobactin ABC-type transport ATP-binding protein; siderophore synthetase component) (Darby *et al.*, 2005). Since iron has been shown to be necessary for the optimal growth of *S. glossinidius* in the previous chapter of this work, it is possible that the siderophore-related genes on this plasmid act for its positive selection by the bacterial host.

4.3.4.2 Plasmid copy numbers *in vitro* and *in vivo*

The copy numbers of the four extrachromosomal elements associated with *S. glossinidius* were higher in bacteria grown in culture than in bacteria *in vivo*. In both situations, pSG3 was the most numerous element, perhaps reflecting the phage-like characteristics of its genes (Darby *et al.*, 2005). This element also covered the broadest range of prevalence, being absent in the *S. glossinidius* GA isolate grown in culture, but present in very high numbers (c. 660 per copy of *SodGroEL*) in the *S. glossinidius* GP isolate.

The low copy numbers of all four extrachromosomal elements in *G. austeni* and *G. p. palpalis* are likely to be due to the greater selective pressures exerted on *S. glossinidius in vivo*. Although growth in medium is not optimal for this

endosymbiotic bacterium, the stresses such as evasion of the host immune system are absent in culture.

Chapter 5

Symbiont and plasmid prevalence in wild and laboratory *Glossina* spp. populations

5 Symbiont and plasmid prevalence in wild and laboratory *Glossina* spp. populations

5.1 Introduction[2]

5.1.1 Detection of bacteria in insects

Prior to the advent of PCR technology, the detection of bacteria in insects was mainly carried out using serological and cell culture techniques. Specific staining techniques may be confounded by the complex microbial flora of the insect, while enzyme-linked immunosorbent assays (ELISA) and immunofluorescent antibody assays (IFA) are prone to the vagaries of individual investigators' antisera preparation and so results from these assays may not be reproducible (Higgins & Azad, 1995). The use of cell culture can lack sensitivity for low numbers of bacteria. PCR techniques have overcome many of these disadvantages to give reproducible, sensitive identification of even non-culturable bacteria from insects. Given that many bacterial symbionts of insects are extremely fastidious and therefore very difficult to isolate or culture *in vitro*, the use of PCR has been fundamental in the identification and molecular characterisation of these bacteria.

Different methods have been used to screen flies for *S. glossinidius* infection, including PCR detection (O'Neill *et al.*, 1993), dot blots with radioactive probes (Welburn & Gibson, 1989) and electron microscopy (Maudlin & Ellis, 1985; Moloo & Shaw, 1989).

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[2] The work presented in this chapter has formed part of a published paper Matthew, C. Z., Darby, A. C., Young, S. A., Hume, L. H. and Welburn, S. (2005). The rapid isolation and growth dynamics of the tsetse symbiont *Sodalis glossinidius*. *FEMS Microbiology Letters* **248**(1): 69 – 74.

5.1.2 Prevalence of secondary symbionts in insects

Secondary endosymbionts exhibit a relatively labile relationship with their hosts in comparison to primary endosymbionts. The facultative nature of secondary endosymbiosis is reflected in the highly variable prevalence of infection in host populations. For example, aphids can contain several different secondary symbionts which show extreme differences in their prevalence, as well as their morphology and tropism, between both diverse species and lineages of their hosts (Fukatsu *et al.*, 2001; Sandstrom *et al.*, 2001).

Chen and Purcell (Chen & Purcell, 1997) found that 80% of clones of pea aphid, collected from a wide geographical area in California, were infected with PASS (Pea Aphid Secondary Symbiont), while approximately 50% of the pea aphids sampled from the same region were positive for a second symbiotic bacterium, PAR (Pea Aphid Rickettsia) (Chen *et al.*, 1996).

It has been suggested that *S. glossinidius* is absent from *G. f. fuscipes* (O'Neill *et al.*, 1993), however, the primers used were specific for an extrachromosomal element of the symbiont which may have been absent. During the course of this study *S. glossinidius* has been successfully isolated from a laboratory colony of *G. f. fuscipes* (Matthew *et al.*, 2005). Species of *Glossina* that are infected with *S. glossinidius* have been found to have varying tropism of the bacterium (Cheng & Aksoy, 1999; Geiger *et al.*, 2005).

The prevalence of *S. glossinidius* has been measured in several different populations and species of tsetse. Using electron microscopy, Moloo and Shaw (Moloo & Shaw, 1989) observed rickettsia-like organisms (RLOs) in 100% of individuals in *Glossina morsitans centralis*. These bacteria have since been taxonomically classified as *S. glossinidius* (Dale & Maudlin, 1999). Geiger *et al.* (Geiger *et al.*, 2005) detected an

extrachromosomal element of *S. glossinidius* in 100% of both *G.palpalis gambiensis* and *G. m. morsitans* individuals analysed by PCR. Studies of three species of *Glossina* from Liberia showed a wide variation in the prevalence of *S. glossinidius* infection (Maudlin *et al.*, 1990). *G.nigrofusca* was found to have a prevalence of 85% *S. glossinidius*-infection, *G. p. palpalis* showed a 9.3% prevalence and 31% of *G.pallicera* sampled were positive for *S. glossinidius* using dot blots with radioactive probes.

5.1.3 *Wolbachia*

Wolbachia spp. have been found in all the major orders of insects (Werren *et al.*, 1995). *Wolbachia* spp. infections have been detected in insects by a variety of methods, including electron microscopy (Binnington & Hoffmann, 1989), DAPI staining (O'Neill & Karr, 1990) and PCR assays (O'Neill *et al.*, 1992).

Previous studies into the prevalence of *Wolbachia* spp. infections in *Glossina* spp. have found a widely ranging prevalences of infection. Cheng *et al.* (Cheng *et al.*, 2000) detected *Wolbachia* spp. in 100% of individuals in the *morsitans* and *fusca* group flies, in sharp contrast to the 0% infection found in the *palpalis* group flies.

In wild flies, Cheng *et al.* (Cheng *et al.*, 2000) found that two thirds of the sampled populations were uninfected by *Wolbachia* spp.. The four populations in which *Wolbachia* spp. was detected showed great variation in the percentage of infected individuals, with infection rates ranging from 11% to 98%. Significant heterogeneity of infection was also found between different species of *Glossina* collected from the same field site. Whereas *G. brevipalpis* from South Africa were uninfected, *G. austeni* from the same site had infection rates of 98%.

5.1.4 Plasmids

Plasmids are semi-autonomous, extrachromosomal circular DNA molecules that are distinct from the normal bacterial genome. There are able to replicate independently of the host cell but often replicate in line with their host in order to ensure the distribution of plasmids into both daughter cells (Bingle & Thomas, 2001). Plasmids are commonly non-essential under non-selective conditions, and the high stability of most low-copy-number plasmids under non-selective growth conditions suggests that these plasmids possess mechanisms by which they are able to ensure their stable maintenance (Zielenkiewicz and Cegłowski, 2001)

The persistence of plasmids in bacterial host populations can be due to a number of factors. Positive selection will act to maintain the plasmid if it is able to increase the fitness of the bacterial host cell through conferring a phenotypic advantage. This is most commonly observed in bacterial populations that carry plasmids conferring resistance to one or more antibiotics (Burrus & Waldor, 2004). Maintenance of such plasmids is associated with a fitness cost and so, in the absence of antibiotic challenge, the plasmid is gradually lost from the population.

Highly efficient vertical transmission of plasmids from parent to daughter cell would also ensure the stability of a plasmid in a population. In the absence of efficient vertical transmission, horizontal transfer of the plasmid between bacteria would ensure the 'infection' of individuals who had not received any plasmids due to uneven distribution at cell division. Some low-copy-number plasmids encode partitioning systems to facilitate equal distribution of plasmids to daughter cells while others even have post-segregational killing systems which eliminate bacterial cells that do not carry any plasmids (Bingle & Thomas, 2001).

Plasmids are found almost ubiquitously in bacterial populations and have a profound effect on the adaptation and evolution of their hosts (Bergstrom *et al.*, 2000). Plasmids are able to increase the fitness of bacteria both on an individual basis and population-wide. The transfer of locally adapted genes to novel strains that have yet to adapt to the environmental conditions would help to preserve such beneficial adaptations (Bergstrom *et al.*, 2000). Plasmids may also act to amplify chromosomally encoded genes in order to increase the expression of certain genes. This is seen in some cases of *Buchnera aphidicola* which is responsible for the provision of certain amino acids to its aphid host (Sobecky *et al.*, 1996). Genes for the synthetic pathways of these amino acids are transferred to plasmids in the bacterial cell which are present at high copy numbers. However, high-copy-number plasmids place a metabolic burden on their host cell that means they are unstable in the absence of strongly selective conditions.

5.1.5 Role of *S. glossinidius* in tsetse susceptibility to trypanosome infection

The prevalence of *S. glossinidius* in wild populations of tsetse flies has been of particular interest since Maudlin (Maudlin, 1982) established that tsetse susceptibility to midgut infection by trypanosomes is a maternally inherited characteristic which Maudlin and Ellis found to be associated with the presence of RLOs in the fly (Maudlin & Dukes, 1985; Maudlin & Ellis, 1985). The relationship between this bacterium and trypanosome infections of tsetse has been investigated in several studies (Maudlin *et al.*, 1990; Welburn *et al.*, 1993).

It is thought that the action of a chitinase, produced by *S. glossinidius*, breaks down chitin during pupation, causing the build up of *N*-acetyl-D-glucosamine (Glc*N*Ac) (Welburn *et al.*, 1993). This compound acts as an inhibitor of tsetse lectins, which

play a role in the immune system of the fly and have been shown to be important in the prevention of trypanosome infection (Maudlin & Welburn, 1987).

5.1.6 Aims

In the present work, the prevalence of *S. glossinidius* was investigated in several populations of *Glossina* from both laboratory colonies and wild populations. In order to assess the stability and maintenance of the extrachromosomal elements associated with *S. glossinidius*, their prevalence was also measured.

The association between *Glossina* spp. and *Wolbachia* is poorly understood, yet this bacterium may have a role in any future attempts to control trypanosomiasis through paratransgenesis. The prevalence of *Wolbachia* infection in wild tsetse was investigated over time in order to give an indication of the population dynamics of this infection.

5.2 Results

5.2.1 Determination of specificity of primers

The specificity of the *SODgroEL* primers for *S. glossinidius* was confirmed by sequencing PCR products derived from tsetse DNA and testing the primers for mis-priming against related bacterial species: *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Figure 5.1). No false positives were observed when these primers were used on closely related bacterial species.

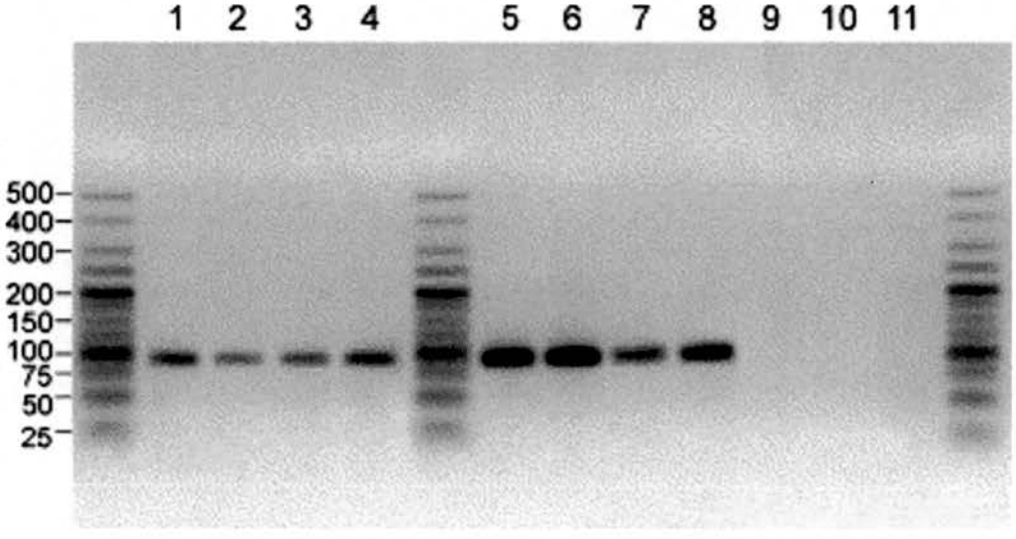


Figure 5.1: Diagnostic PCR with *S. glossinidius*-specific groEL primers. Template DNA from four species of tsetse fly (1–4) and liquid cultures of four strains of *S. glossinidius* (5–8). Lane 1: *G. m. morsitans*; 2: *G. austeni*; 3: *G. f. fuscipes*; 4: *G. p. palpalis*; 5: *S. glossinidius* GM; 6: *S. glossinidius* GA; 7: *S. glossinidius* GF; 8: *S. glossinidius* GP. Negative controls using template DNA from related bacterial taxa, lane 9: *E. coli*; 10: *K. pneumoniae* and 11: *P. aeruginosa*. DNA ladder: Bioline HyperLadder V, 25–500 bp.

PCR using the *SODgroEL* primers on DNA from various *Glossina* spp. consistently yielded single products of the expected length of 95bp. Specific PCR using these primers confirmed that all clones isolated from tsetse were *S. glossinidius*. The bacterium was detected in both whole fly and bacterial culture DNA templates from different tsetse species.

The specificity of these primers for use on tsetse material was verified by sequencing products amplified from tsetse DNA templates; in all cases the sequences obtained were identical to the *S. glossinidius* genes present in the GenBank database (NCBI non-redundant database blastn server).

5.2.2 Screening of wild flies for *S. glossinidius* and Plasmids 1 to 4

The percentage prevalence of *S. glossinidius* and its four associated extrachromosomal elements was measured in populations of wild flies from Tanzania and Zimbabwe (Table 5.1). No significant difference was found between the prevalence of *S. glossinidius* and that of the four extrachromosomal elements in either *G. austeni*, *G. m. morsitans* or *G. pallidipes*. The difference in percentage prevalence was found to be significant between *G.morsitans* and *G. pallidipes* ($p = 0.036$). The prevalence of infection in these three species ranged from 16 – 30%. The prevalence of *S. glossinidius* infection in *G. brevipalpis* was found to be 100%. This was significantly higher ($p < 0.001$) than *S. glossinidius* prevalences in the other three species of *Glossina* screened in this study. All *G. brevipalpis* screened were found be positive for *S. glossinidius* infection. In this species of *Glossina* neither pSG3 nor pSG4 were detected.

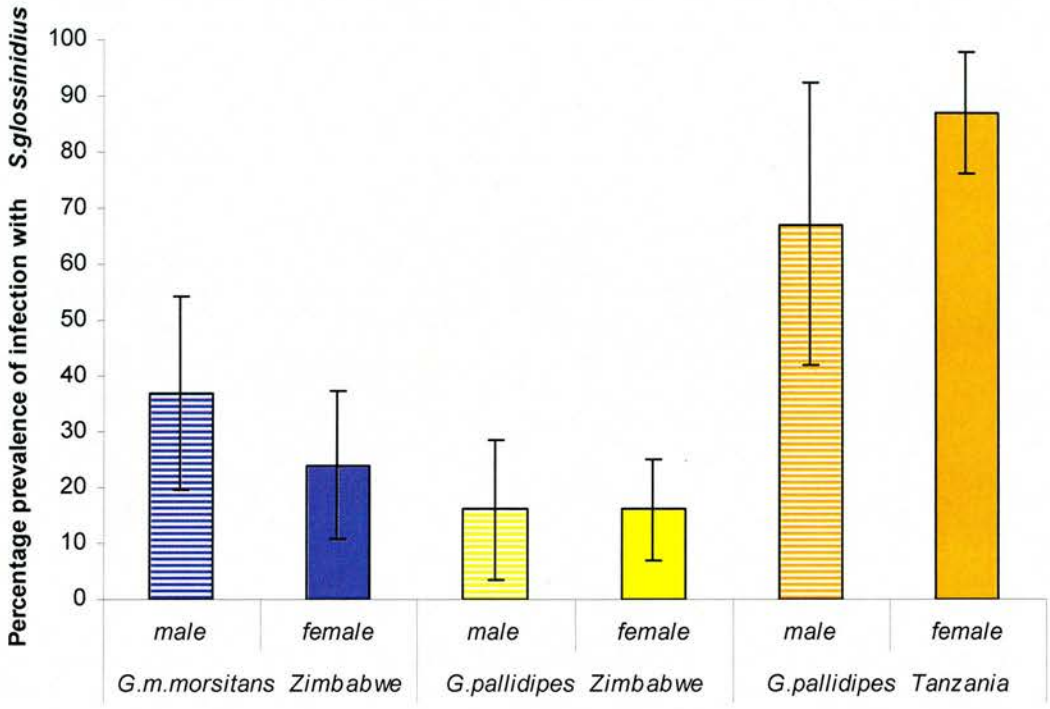
Country	*Glossina* spp.	Percentage prevalence				
		S. glossinidius	pSG1	pSG2	pSG3	pSG4
Tanzania	*G. austeni* [n=52]	17	15	17	17	19
	G. brevipalpis [n=17]	100	94	100	0	0
Zimbabwe	*G. m. morsitans* [n=71]	30	30	28	32	31
	G. pallidipes [n=94]	16	15	15	16	15

Table 5.1: Percentage prevalence of *S. glossinidius* and its associated extrachromosomal elements in different species of *Glossina* collected from Tanzania and Zimbabwe.

5.2.3 *S. glossinidius* in *G. m. morsitans* and *G. pallidipes* wild flies at two different time points

The presence of *S. glossinidius* was investigated in wild populations of *G. m. morsitans* and *G. pallidipes* flies collected from Tanzania and Zimbabwe at two time points, one year apart.

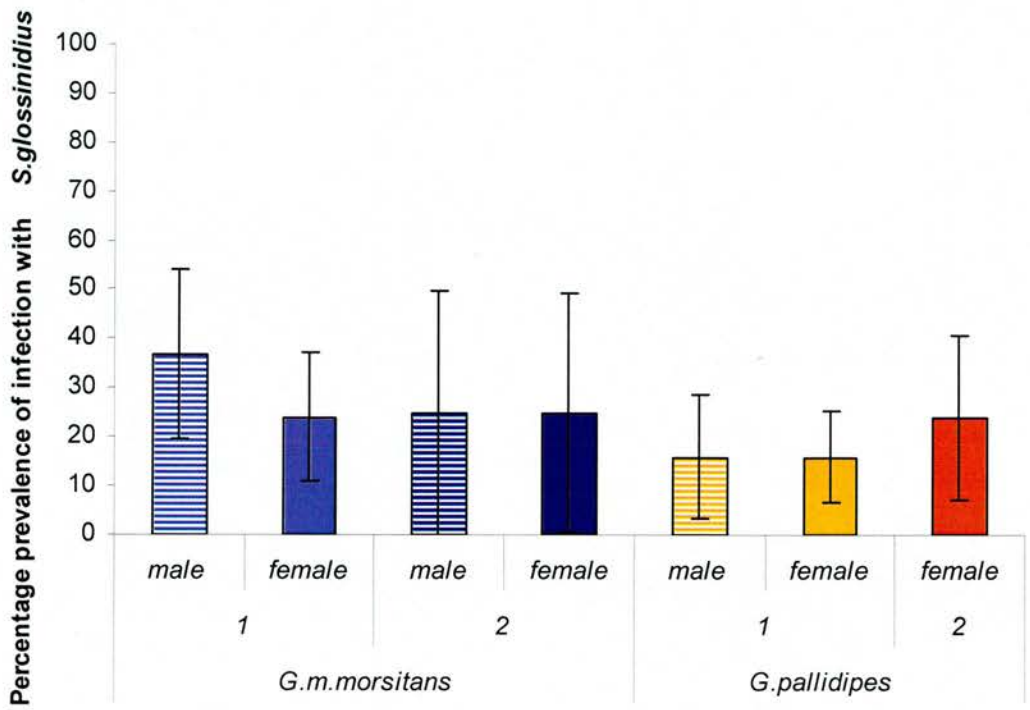
G. m. morsitans was found to have a prevalence of infection with *S. glossinidius* of 37% in male flies (n = 30) and 24% in female flies (n = 41) (Graph 5.1). *G. pallidipes* flies collected from Zimbabwe had a prevalence of infection with *S. glossinidius* of 16% in both male (n = 32) and female (n = 62) flies, while those collected from Tanzania had prevalences of 67% in male flies (n = 15) and 87% in female flies (n = 75).



Graph 5.1: Prevalence of *S. glossinidius* infection in wild populations of *G. m. morsitans* and *G. pallidipes* samples from Zimbabwe and Tanzania. Error bars show 95% confidence intervals.

The prevalence of *S. glossinidius* infection was found to be significantly lower in Zimbabwe than in Tanzania ($\chi^2_{1,368}$=34.84, p <0.001) at the first sampling time point. *G. m. morsitans* was found to have a lower prevalence of infection than *G. pallidipes* ($\chi^2_{3,365}$=58.51, p <0.001). There was no significant effect of fly sex on the prevalence of *S. glossinidius* infection ($\chi^2_{1,364}$=0.12, p = 0.728).

No flies were collected from Tanzania at the second sampling time point, so the Zimbabwean data set was reanalysed separately (Graph 5.2). At the second sampling time point *G. m. morsitans* was found to have a prevalence of infection with *S. glossinidius* of 25% in both male (n = 8) and female (n = 12) flies. *G. pallidipes* was found to have a prevalence of 24% in female flies (n = 25). No male *G. pallidipes* were collected at the second sampling time point.

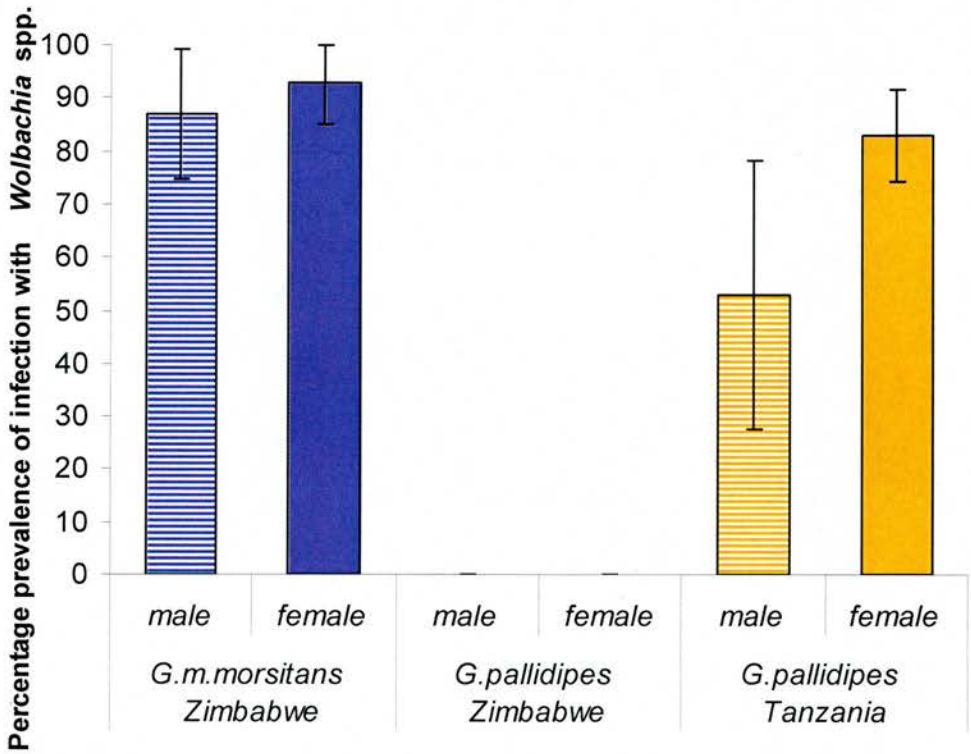


Graph 5.2: Prevalence of *S. glossinidius* infection in wild populations of *G. m. morsitans* and *G. pallidipes* samples collected from Zimbabwe at two time points (1 – first time point, 2 – second time point). Error bars show 95% confidence intervals.

The prevalence of *S. glossinidius* infection did not change significantly in the time between fly collections ($\chi^2_{1,206} = 0.195$, $p = 0.659$). There were no significant differences in the prevalence of *S. glossinidius* between fly species ($\chi^2_{1,208} = 3.52$, $p = 0.061$) or sex ($\chi^2_{1,207} = 0.28$, $p = 0.597$).

5.3 Screening of wild *G. pallidipes* and *G. m. morsitans* for *Wolbachia* spp.

The presence of *Wolbachia* spp. was investigated in wild populations of *G. m. morsitans* and *G. pallidipes* flies collected from Tanzania and Zimbabwe. *G. m. morsitans* was found to have a prevalence of infection with *Wolbachia* spp. of 87% in male flies (n = 30) and 93% in female flies (n = 41) (Graph 5.3).



Graph 5.3: Prevalence of *Wolbachia* spp. infection in wild populations of *G. m. morsitans* and *G. pallidipes* from Zimbabwe and Tanzania. Error bars show 95% confidence intervals.

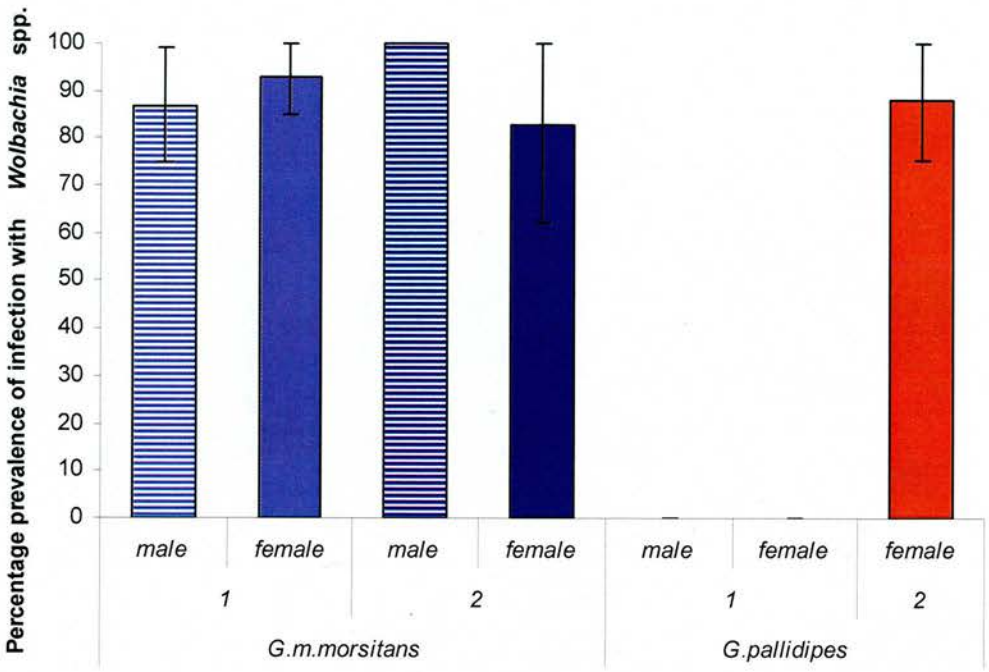
No *Wolbachia* spp. infections were detected in either male (n = 32) or female (n = 62) *G. pallidipes* flies collected from Zimbabwe. *G. pallidipes* collected from

Tanzania had prevalence of *Wolbachia* spp. infection of 53% in male flies (n = 15) and 83% in female flies (n = 75).

The prevalence of *Wolbachia* spp. infection was found to be significantly lower in Zimbabwe than in Tanzania ($\chi^2_{1,253} = 37.12$, $p < 0.001$) at the first sampling time point (Graph 5.3). *G. pallidipes* was found to have a lower prevalence of infection than *G. m. morsitans* ($\chi^2_{1,252} = 174.65$, $p < 0.001$). Male flies of either species were found to have a lower prevalence of infection than female flies ($\chi^2_{1,251} = 5.58$, $p = 0.02$). When the data were reanalysed excluding the Zimbabwe *G. pallidipes* subset the significant differences in prevalence of *S. glossinidius* infection remained constant with a higher prevalence in *G. pallidipes* than in *G. m. morsitans* ($\chi^2_{1,159} = 4.55$, $p = 0.033$) and a higher prevalence in female flies than in males ($\chi^2_{1,158} = 5.58$, $p = 0.018$).

At the second sampling time point *G. m. morsitans* was found to have a prevalence of infection with *Wolbachia* spp. of 100% in male flies (n = 8) and 83% in female flies (n = 12). *G. pallidipes* was found to have a prevalence of 88% in female flies (n = 25). No male *G. pallidipes* were collected at the second sampling time point.

No flies were collected from Tanzania at the second sampling time point, so the Zimbabwean data set was reanalysed separately (Graph 5.4). The main effects of species and sex retained the same relationship, with *G. m. morsitans* having a higher prevalence of infection than *G. pallidipes* ($\chi^2_{1,208} = 118.45$, $p < 0.001$) and female flies having a higher prevalence than male flies ($\chi^2_{1,207} = 7.56$, $p = 0.006$). In addition the prevalence of *Wolbachia* spp. infection in flies collected at sampling time point 2 was significantly higher than that at sampling time point 1 ($\chi^2_{1,206} = 67.02$, $p < 0.001$). A significant interaction between species and time point was also found ($\chi^2_{1,205} = 21.03$, $p < 0.001$).



Graph 5.4: Prevalence of *Wolbachia* spp. infection in wild populations of *G. m. morsitans* and *G. pallidipes* samples at two time points. Error bars show 95% confidence intervals.

5.3.1 Screening of laboratory *G. brevipalpis* for *S. glossinidius* and Plasmids 1 to 4

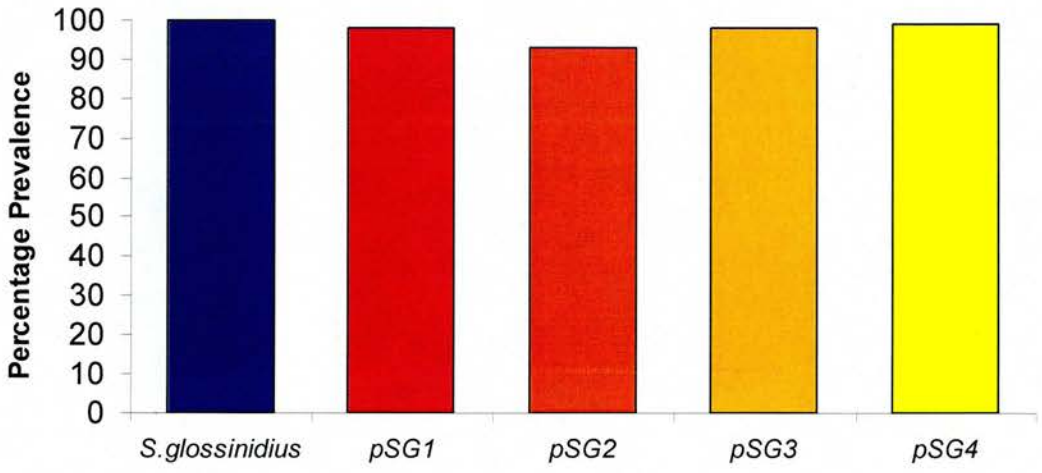
The prevalence of *G. brevipalpis* infection with *S. glossinidius*, and its associated extrachromosomal elements, was determined for a laboratory colony of flies as well as a sample of wild flies collected from Tanzania (Table 5.2). 100% of the wild flies tested (n = 17), and 98% of the laboratory colony individuals (n = 48) were found to be infected with the bacterium. The significance of these results was analysed using Fisher's exact test, with a two-tailed P-value considered to be significant at <0.05. There was no significant difference between the prevalence of *S. glossinidius* in the two populations (p = 1.000). The prevalence of pSG1 and pSG2 did not differ significantly from the prevalence of *S. glossinidius* in either wild or lab flies. No instances of pSG3 or pSG4 were detected within the wild fly population. The prevalence of these two plasmids in the laboratory colony flies was also significantly lower than that of *S. glossinidius* infection (p <0.001; p <0.001 respectively).

***G. brevipalpis* population**	**Percentage prevalence**				
	S. glossinidius	**pSG1**	**pSG2**	**pSG3**	**pSG4**
Wild (Tanzanian) [n = 17]	100 [b]	94	100	0 [a b]	0 [b]
Laboratory colony [n = 48]	98 [c]	90	79	63 [a c]	8 [c]

Table 5.2: Percentage prevalence of *S. glossinidius* and its extrachromosomal elements in two populations of *G. brevipalpis*. The letters [a], [b] and [c] denote statistically significant differences.

5.3.2 Screening of *S. glossinidius* isolated from G. brevipalpis for plasmids 1 to 4

Cultures of bacteria isolated from laboratory colonies of *G. brevipalpis* were confirmed as *S. glossinidius* by PCR with *SODgroEL* primers (Graph 5.5). The prevalence of the four extrachromosomal elements associated with *S. glossinidius* was determined and the results were analysed using Fisher's exact test, with a two-tailed p-value considered to be significant at <0.05. The extrachromosomal element pSG2 was found to be absent in a significant number of isolates ($p = 0.014$). There was no significant difference in the prevalence of the other three extrachromosomal elements, pSG1 ($p = 0.497$), pSG3 ($p = 0.497$) or pSG4 ($p = 0.999$) compared to *S. glossinidius*.



Graph 5.5: Prevalence of the extrachromosomal elements of *Sodalis glossinidius* in cultures isolated from laboratory colony *Glossina brevipalpis* flies.

5.3.3 Screening of *G. m. morsitans* fly urine and faeces for viable *S. glossinidius*

Bacteria were cultured from the urine of 9 of the 14 individual tsetse flies sampled. No bacterial growth was obtained from faecal samples from the same flies, even after prolonged incubation. Of the samples from which bacteria were isolated, 7 had between 1 and 20 viable cell forming units (CFUs) and 2 had over 50 CFUs, at which point the individual colonies became uncountable (Table 5.3). The data was found to be aggregated using a test of the index of dispersion ($\chi^2_{13} = 365.99$, $p < 0.001$).

Groups	Frequency
No growth	5
1 to 49 cfu	7
>50 cfu	2

Table 5.3: Colony forming units isolated from *G. m. morsitans* urine from 14 individual flies.

The bacterial colonies had the same phenotype as *S. glossinidius* – round, pale grey colonies of <1 mm in diameter. The bacteria were screened by PCR with *S. glossinidius* 16s rDNA primers to confirm their identity (Figure 5.2). All isolates tested positive for *S. glossinidius* 16s rDNA. These results were confirmed by sequencing PCR products amplified from the bacterial templates. In all cases the sequences obtained were identical to the *S. glossinidius* genes present in the GenBank database (NCBI non-redundant database blastn server).

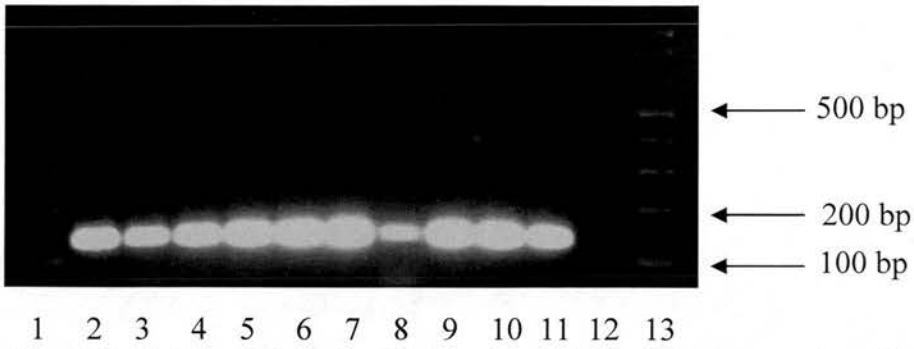


Figure 5.2: Gel electrophoresis of PCR for *S. glossinidius* 16S primers on bacterial growth from *G. m. morsitans* urine. Lanes 1 and 13 – 100 bp molecular weight marker; lanes 2 to 10 – bacterial samples from individual flies; lane 11 –*S. glossinidius* GM DNA positive control; lane 12 – H_2O negative control.

5.4 Discussion

The results of this study show that *S. glossinidius* is present in wild populations of *Glossina* spp. at widely varying prevalences. This finding is in accordance with Maudlin *et al.* (Maudlin *et al.*, 1990) who showed that the prevalence of *S. glossinidius* in Liberian wild tsetse flies ranged from c.10 – 85% across three different species of tsetse. The significantly higher prevalence, found in this work, of *S. glossinidius* in *G. pallidipes* collected from Tanzania, as compared to those collected from Zimbabwe, shows that the variation in prevalence is not purely species-specific.

Populations of *G. pallidipes* are known to be very different in terms of susceptibility to trypanosome infection (Moloo, 1993) and genetic differentiation (Langley *et al.*, 1984; Gooding & Krafsur, 2004). The historical record shows that a rinderpest epizootic, beginning in 1887, killed a great many mammalian hosts, causing bottlenecks in southern populations of *G. pallidipes* and *G.m.centralis* (Ford, 1971). As *S. glossinidius* is maternally inherited, current prevalences of infection in populations that were previously affected by severe bottlenecks would be influenced by the 'founder effect'. This term more usually refers to the genetic drift observed in a population which has arisen from a restricted number of individuals, but in this instance it would apply to the effect that the symbiotic status of the founding flies would have on the prevalence of symbiotic infection in the resulting population.

The prevalence of tsetse fly infection with *S. glossinidius* is very different between laboratory colonies and wild populations, with the former showing a significantly higher prevalence than the latter. Using electron microscopy, Moloo and Shaw (Moloo & Shaw, 1989) observed RLO in 100% of individuals within their laboratory colony of *Glossina morsitans centralis*. In 1989 the *G. m. morsitans* laboratory colony at Langford House, University of Bristol (20 years after it was first

established) was found to have a prevalence of infection with *S. glossinidius* of 80% (Baker *et al.*, 1990). Flies sampled at the same time, from the wild populations from which the Langford colony was derived, had a prevalence of 20%.

The partial infection of host populations by secondary endosymbionts has been suggested to be an indication that the relationship between bacterium and host is of a neutral or parasitic nature (Fukatsu *et al.*, 2001). It has also been hypothesised that low secondary symbiont prevalences are an indicator of the shorter length of time of co-adaption compared to primary symbionts and their hosts (Fukatsu *et al.*, 2000). Fine (Fine, 1975) stated that, "for a strictly matroclinal infection to persist in a population it must give some selective advantage". The presence of *S. glossinidius* in wild populations of different species of *Glossina* suggests that the bacterium does indeed "persist", raising the question of what positive effects *S. glossinidius* has upon its host.

S. glossinidius infection has been found to have a small beneficial effect on tsetse (Baker *et al.*, 1990) with laboratory studies indicating that infection with *S. glossinidius* corresponds to a 5% increase in pupal survival rate. However, *S. glossinidius* is also linked to an increase in susceptibility of *Glossina* spp. to trypanosome infection (Maudlin & Ellis, 1985). Trypanosome infection places a burden on tsetse flies that may lead to a loss of flight energy (Bursell, 1981), increased mortality (Nitcheman, 1988) and poor nutritional status (Ryan, 1984).

'Non-beneficial' symbionts may be maintained at a low frequency in host populations, as seen in the case of the *Spiroplasma* secondary symbiont of *Acyrthosiphon pisum*. Despite the evidence of negative fitness effects of this symbiont on its host, as well as the imperfect transmission rate, the infection is seen to persist in host populations (Fukatsu *et al.*, 2001). Whilst the exact mechanism by which it is maintained is unknown, it is possible that *Spiroplasma* may be able to

confer benefits to its host under specific environmental conditions, or that the symbiont is capable of both vertical and horizontal transmission between individuals. In the same way, it is possible that *S. glossinidius* may have a beneficial effect on the tsetse fly under environmental stresses that are not encountered in a laboratory setting.

From the present work, it is clear that the possibility of horizontal transfer as a means of maintenance of this bacterium within its host population is very real. *S. glossinidius* was cultured from urine taken from flies at their first feed. During the normal feeding regime of laboratory colony flies, there is urination on the silicone membrane through which the flies feed. This provides occasion for urine from one individual to contaminate the blood meal of another as the proboscis may pass through depositions of urine during feeding. This could explain the ubiquity of *S. glossinidius* infections in laboratory colonies as opposed to the low level of infection in most wild populations of *Glossina* spp.

The absence of viable bacteria in the faecal samples may be explained by the relatively harsh conditions of this medium as compared to the urine of the fly. Any bacteria that might be present in the faeces would have been exposed to the contents of the blood meal in the gut, prolonged digestive activity and extremely arid conditions in the faecal droplet. It has also been noted that Gram-negative bacteria are particularly sensitive to desiccation, with complete loss of viability in cells of *E. coli* and *Salmonella typhimurium* in a dry atmosphere after seven hours (Hirai, 1991).

It may be that *S. glossinidius* is able to enter a viable-but-non-culturable phase in order to survive in harsh conditions, as has been suggested previously (Matthew *et al.*, 2005). This physiological state has been reported in several enterobacteria, such as *Campylobacter jejuni*, *E. coli* and *Vibrio cholerae* (Tholozan *et al.*, 1999) (Xu *et al.*, 1982). These bacteria commonly enter this state when under environmental

stress. In order to establish whether or not this is the case it would be appropriate to screen the faecal sample by PCR for the presence of S. glossinidius DNA and to stain the sample using the *Bac*Light™ viability analysis system. Using this system, large populations of small, healthy *S. glossinidius* cells were observed in two month old stationary phase liquid cultures (Matthew *et al.*, 2005).

The establishment of symbiotic infection after ingestion of bacteria in the blood meal has been experimentally shown by Dotson (2003). Transformed *Rhodococcus rhodnii* were added to the blood meal of *Rhodnius prolixus* as first instar nymphs and were found to persist in the adult insect. The natural route of transmission of this symbiont is via coprophagous activity of the insect. It is possible that *S. glossinidius* is able to infect its host through urophagous behaviour of the tsetse fly as tsetse urinate almost immediately after feeding (Bursell, 1960).

It has been suggested that vertical transmission of secondary symbionts in aphids may not be 100% perfect (Fukatsu *et al.*, 2000) which raises questions as to how symbionts are maintained in natural host populations. If the relationship is non-beneficial, and does not increase the fitness of the host, then it would be expected that there would be negative selective pressure against the symbiont and it would gradually disappear from the host population. In light of the persistence of such relationships in natural populations over time, it is possible that horizontal transmission is the mechanism by which infection is maintained. However, in laboratory colonies of aphids there have been no observed instances of natural horizontal transfer (Chen & Purcell, 1997; Sandstrom *et al.*, 2001).

Russell & Moran (Russell & Moran, 2005) have recently studied horizontal transfer of bacterial symbionts in aphids by means of microinjection of the bacteria into novel hosts. Their study found that, although new infections may be prevented by

"negative fitness effects and low transmission efficiency", in some cases at least the establishment of a symbiosis in a novel host was successful.

Horizontal transmission between hosts has also been suggested to account for the lack of genome sequence diversion between secondary symbionts infecting different species of host (Fukatsu *et al.*, 2000; Sandstrom *et al.*, 2001). The common restriction of secondary symbionts to just one host species suggests that any horizontal transmission takes place more commonly within than between species (Aksoy *et al.*, 1997). This may be due to limited opportunities for interspecies transmission, limited host ranges in which the bacteria is able to survive or bacterial death due to maladaptive phenotype effects upon invasion of a novel host (Russell *et al.*, 2003).

Analysis of the 16S rDNA sequences of *S. glossinidius* has shown isolates of the bacterium to be extremely homologous, even between widely divergent subgenera of the tsetse host (Aksoy *et al.*, 1997). This homology has been hypothesised to be a result of either recent invasions of *Glossina* spp. either by means of independent acquisitions or horizontal transmission of the bacterium (Aksoy *et al.*, 1997). Phylogenetic analysis of the *fusca* group species, including *G. brevipalpis*, has shown that they form a distinct group from the sister groups of *morsitans* and *palpalis* species (Chen *et al.*, 1999). These relationships were echoed in the analysis of the primary symbionts (Chen *et al.*, 1999).

Whilst the extrachromosomal elements of *S. glossinidius* were uniformly present in *G. austeni*, *G. m. morsitans* and *G. pallidipes* wild flies, two of the extrachromosomal elements of *S. glossinidius*, pSG3 and pSG4, were found to be entirely absent in the population of wild *G. brevipalpis* and present at a significantly lower level in laboratory colony *G. brevipalpis*. The presence of pSG3 and pSG4 in the laboratory colony of flies may be explained by the possible horizontal

transmission of *S. glossinidius* between different species of *Glossina* during artificial feeding. These elements may have been present in *G. brevipalpis* at a level below the sensitivity of this diagnostic PCR, or it may be that the sequences are sufficiently divergent to render the assay ineffective. In particular, pSG3 has been found to be larger in *S. glossinidius* isolated from *G. m. morsitans* than from *G. austeni* or *G. p. palpalis* (S. Young, personal communication).

The difference in prevalence of the four extrachromosomal elements of *S. glossinidius* in bacterial isolates from *G. brevipalpis* to that of bacteria assayed *in vivo* from both wild and laboratory colony populations may be explained by the extreme differences between bacterial growth conditions in the two situations. The isolation and *in vitro* growth of any bacterial colony will automatically select for a different set of fitness requirements than would be needed for growth *in vivo* (Fux *et al.*, 2005).

Bacteria that can be cultured axenically are those that have the requisite characteristics for growth under these conditions. This may explain why the isolates of *S. glossinidius* from *G. brevipalpis* laboratory colony flies were found to have a full complement of four extrachromosomal elements, when screening of the bacteria *in vivo* revealed that pSG3 and pSG4 were retained by a relatively small proportion of the bacterial population. If these plasmids encode factors that are necessary for growth *in vitro* then only bacteria which have retained them will be isolated.

Bacterial strains may also diverge from their naturally occurring counterparts through the course of sequential passage *in vitro*. This has been observed to lead to non-random genetic alterations that increase the fitness of the bacteria in the novel environment, with resultant changes to the original phenotype (Edwards *et al.*, 2002).

5.4.1 *Wolbachia* spp. in *G. m. morsitans* and *G. pallidipes* at two different time points

The screening of wild populations of *G. m. morsitans* and *G. pallidipes*, collected from Zimbabwe, has shown two disparate dynamics of *Wolbachia* spp. infection. In the *G. m. morsitans* population, approximately 90% of flies are infected with *Wolbachia*. This prevalence is seen to remain stable as there was no significant change in the proportion of infected flies when the same population was sampled a year later. In contrast, the prevalence of infection in *G. pallidipes* is seen to increase from 0 to 80% over the course of the year. This spread of infection is indicative of the production of cytoplasmic incompatibility (CI) by *Wolbachia* spp. This is because, in CI-producing infections, infected females will on average produce more viable progeny than uninfected females as the former are 'immune' to the effects of CI. This was first noted in mosquitoes by Caspari & Watson (Caspari & Watson, 1959). The severity of the effects of CI on the host's reproduction are proportional to the speed of the spread in populations (Turelli & Hoffmann, 1999).

The spread of *Wolbachia* infections has only been tracked in a limited number of insects. In wild populations of *Drosophila simulans* the infection has spread northwards throughout Central and Northern California over ten years (Turelli & Hoffmann, 1995). In an earlier study by Turelli & Hoffmann (Turelli & Hoffmann, 1991), the Riverside strain of *Wolbachia* was found to be spreading at a rate of more than 100 km per year through wild populations of *D. simulans*, increasing prevalences of infection from very low to almost 100% within three years.

In the light of these studies, the rate of increase in prevalence of infection in the *G. pallidipes* population studied appears extreme. However, several factors may affect the results of this study. Firstly, the method of DNA extraction from flies collected at the two time points in this study were different; the first method being CTAB

extraction and the second using a spin column (Qiagen, UK). The two methods may yield different qualities of DNA, affecting the sensitivity of the PCR screening for *Wolbachia*. This is unlikely, however, as neither the prevalence of *Wolbachia* nor that of *S. glossinidius* was seen to increase significantly in samples that had been extracted using the spin column protocol. In addition the sample size of the *G. pallidipes* population collected at the second time point was smaller than the first and consisted only of female flies.

The generation time of *Glossina* spp. is relatively slow, approximately 43 days, due to their unusual viviparous method of reproduction (Gooding & Krafsur, 2005). Over the course of a year this would amount to around 8 generations of flies. This seems to be inconsistent with the rapid increase in the presence of *Wolbachia* spp. in *G. pallidipes* in Zimbabwe from 0% to 80% over the course of one year.

As laboratory colonies of *Glossina* spp. have been found to exhibit either 100% prevalence of *Wolbachia* spp. infection or 0%, it has not so far been possible to investigate whether *Wolbachia* causes any reproductive abnormalities in tsetse flies (Cheng *et al.*, 2000). If this bacterium did not have any effects on the reproductive system of tsetse, then its maintenance and spread through the population could potentially be explained by either horizontal transmission or beneficial interactions with the host organism, as seen in the nematode-*Wolbachia* symbiosis (Stouthamer *et al.*, 1999).

The significant difference that was found in this study between *Wolbachia* infection in male and female flies may reflect the disparate dynamics of this symbiosis in the two sexes. In some studies of *Drosophila* spp. infected females have been shown to maintain a high population of *Wolbachia* throughout their life (Hoffmann *et al.*, 1986). This corresponds with the mode of transmission of *Wolbachia*, which is passed from mother to offspring. In contrast, males have been found to gradually

lose their infective status with age. Maintenance of infection in male flies is a 'dead end' for these bacteria as there is no potential for their transmission. The samples screened here for the prevalence of *Wolbachia* spp. infection were from a spectrum of age groups.

The prevalence of *S. glossinidius* infection in *G. pallidipes* populations in Zimbabwe was observed not to change significantly over time. In this same population of flies the prevalence of *Wolbachia* spp. infection was seen to increase from 0 to 80% over the course of one year. This spread of *Wolbachia* spp. through the population did not affect the prevalence of *S. glossinidius* calling into question the suggestion that *Wolbachia* could be used as a force to drive transgenic *S. glossinidius* into a population of tsetse as a control measure against trypanosomiasis (Cheng & Aksoy, 1999).

Chapter 6

Discussion

6 Discussion

6.1 Growth kinetics and siderophore production

This thesis has concentrated on the growth and prevalence of *S. glossinidius*, the secondary endosymbiont of the tsetse fly. Previously published works had focused on the *S. glossinidius* found in *G.m.morsitans*. This work explores the growth of *S. glossinidius* isolates from tsetse covering the three groups, namely the *fusca*, *palpalis* and *morsitans* groups.

In vitro growth of *S. glossinidius* followed a standard bacterial growth curve but was extended over a period of several days. With the exception of several isolates from *G. brevipalpis* it was found that *S. glossinidius* from the tsetse species examined had, on average, a generation time of approximately 26 hours. In this time frame a single *E. coli*, with a doubling time of 20 minutes, would have increased in number to 2^{72} cells. In the fly, the generation time of *S. glossinidius* was estimated to be 7 hours shorter than that measured *in vitro*. This is likely to be due to the specialisation of this symbiont to growth within its host and the sub-optimal nature of cell free growth conditions. Slow growth rates are characteristic of arthropod-associated symbionts in culture, almost all of which take around four days to reach mid-exponential phase (Darby & Welburn, 2006).

The reduction of incubation temperature of *S. glossinidius* from 26.5˚C to 20˚C had no effect on the growth rate of the bacterium in this study, although previous work had shown that the generation time of *S. glossinidius* incubated at 22 ˚C in cell culture decreased six-fold compared to incubation at 25˚C. In light of the current work, this decrease is postulated to have been due to the effect of the lower

incubation temperature on the insect cell line, indirectly slowing the growth of *S. glossinidius* while in cell culture. Similar reasoning might be also applied to the findings of Welburn & Maudlin (1991), that the incubation of tsetse pupae at lower temperatures resulted in a decrease in the number of *S. glossinidius* in the fly population.

Iron is fundamental to bacterial growth, however, high levels of this ion can promote oxidative stress. The tsetse blood meal contains large amounts of iron which has the potential to oxidise membranes and nucleic acids. In order to overcome these hazardous effects tsetse and other blood-feeding insects have evolved a number of mechanisms to sequester iron. This results in very little iron being available for bacterial growth. The current work shows that *S. glossinidius* produces siderophores, supporting the putative role of siderophore transport and biosynthesis genes found on both the bacterium's chromosome and encoded on the extrachromosomal element, pSG1 (Toh *et al.*, 2006). The production of siderophores was increased in response to growth of the bacterium in iron-deficient medium. Siderophore production by *S. glossinidius* was found to vary throughout the phases of growth of the bacterium *in vitro*, with peaks of production during lag phase and late exponential phase.

6.2 Quantification of *Glossina* spp. symbionts

The growth of *S. glossinidius in vivo* was seen to be highly dynamic over the developmental course of the tsetse, with the highest rates of growth being observed in the early stages of pupal development. In the adult fly, the population size and density of *S. glossinidius* was seen to be maintained at a relatively constant level. This may indicate that the symbiont population is in some way regulated by the host in order to prevent excessive bacterial proliferation. It may also be that the resources

available to *S. glossinidius* are less abundant in the adult fly than in the comparatively less structured pupa.

The population size of *S. glossinidius* was found to vary between different *Glossina* spp. as supported by previous semi-quantitative and qualitative studies (Moloo & Shaw, 1989; Cheng & Aksoy, 1999). *G. brevipalpis* was seen to harbour significantly lower levels of *S. glossinidius* than *G. m. morsitans* despite the larger body size of the former species. The number of *S. glossinidius* in wild flies was several orders of magnitude lower than that measured in laboratory colony flies, probably due to the stresses encountered by tsetse in their natural habitat that are eliminated in the laboratory setting.

A study investigating the prevalence of *S. glossinidius* in trypanosome-infected and -uninfected tsetse populations in Liberia found that flies with *S. glossinidius* infections had significantly higher trypanosome infection rates than flies that were negative for *S. glossinidius* (Maudlin *et al.*, 1990). The association between *S. glossinidius* infection and trypanosome infection has recently been called into question in a study of the symbiont's presence with regard to vectoral competance (Geiger *et al.*, 2005). Geiger *et al.* (2005) found that there was no direct correlation between *S. glossinidius* infection and the ability of the trypanosome to infect the tsetse fly. Similar findings were made by Moloo and Shaw, who observed rickettsia-like organisms in all trypanosome-infected and non-infected midguts of *G.m.centralis* (Moloo & Shaw, 1989). However, these results are inconclusive as the relationship between *S. glossinidius* and susceptibility to trypanosome infection is known to be quantitative (Welburn & Maudlin, 1991). The quantification of *S. glossinidius* populations in infected and uninfected flies in the current work found no difference between the two, which was in accordance with the findings of Rio *et al.* (Rio *et al.*, 2005). However, the point at which the *S. glossinidius* population size is relevant to trypanosome infection is at the level of the teneral fly. This creates problems as it is currently a logistical impossibility to both quantify the symbiont

levels in an individual teneral fly and go on to see if that individual is susceptible to trypanosome infection, as the former experiment necessitates the homogenisation of the entire fly.

The decrease in the range of *S. glossinidius* population in teneral flies compared to fed flies suggests that there may be some event occurring during the time after the first blood meal is taken that regulates the numbers of *S. glossinidius*. A possible explanation of the nature of this regulatory event would be that excessive numbers of *S.glossinidius* are excreted from the midgut immediately after the first feed. The discovery of viable *S. glossinidius* cells in the urine of tsetse after their first blood meal may provide a way to screen the bacterium's population size in an individual fly whilst allowing further experiments to be carried out on that individual. In Chapter 5 of the current work, the number of excreted *S. glossinidius* varied between individuals in a pattern similar to the variation of *S. glossinidius* population levels observed in teneral flies in Chapter 4 of this work.

The current work found that *S. glossinidius* cultured *in vitro* displayed polyploidy, with approximately eight genome copies per bacterial cell. This may result in false results being obtained from the life cycle data obtained in this work, as it is impossible to tell how many genome copies may be present in *S. glossinidius in vivo*. As qPCR is only able to identify the number of copies of the target gene, it can only determine how many gene copies are present in the sample. If *S. glossinidius* present *in vivo* does not display this polyploidy of eight genome copies per bacterial cell, then the enumeration of the symbiont population could be out almost to one order of magnitude.

Further work might go on to examine the copy number of this bacterium at different stages of growth *in vitro* in order to determine whether the polyploidy is a stable

characteristic or if it is a product of rapid cell growth, as seen in the case of some enterobacteria (Zyskind & Smith, 1992).

6.3 Symbiont and plasmid prevalence in wild and laboratory *Glossina* spp. populations

Screening of wild flies showed that unlike lab colonies, the prevalences of the *S. glossinidius* and *Wolbachia* spp. was not always 100%. Differences in *S. glossinidius* prevalence were found between tsetse species in the same areas and between the same species in different areas. The prevalence of *S. glossinidius* in *G. brevipalpis* from Tanzania was 100% while *G. austeni* from the same area was only 20%. A striking difference was also noted between *G. pallidipes* from Tanzania and Zimbabwe, where the prevalence of *S. glossinidius* was almost 80% in the former compared to 10% in the latter species.

The high disparity in the prevalences of *S. glossinidius* in *G. austeni* and *G. brevipalpis* would suggest that no horizontal transmission is taking place between these two species of tsetse. However, analyses could be undertaken in future years to examine whether or not the prevalence of *S. glossinidius* in *G. austeni* fluctuated. In the present work it was shown that viable *S. glossinidius* exist in the urine of the tsetse fly. This could explain the ubiquity of this symbiont in laboratory colonies of tsetse. In the wild it is unlikely that a fly would come into contact with another fly's urine whilst feeding, due to the infinitely increased area over which individuals may feed compared to the laboratory setting.

The present work investigated the copy number of the various extrachromosomal elements of *S. glossinidius*. Although *S. glossinidius* from most species of tsetse

showed the presence of all four elements, *G. brevipalpis* from the wild did not possess either pSG3 or pSG4 and the copy number of these elements was significantly reduced in *G. brevipalpis* lab colony flies. However, subsequent analysis of *S. glossinidius* GB cultured in *vitro* showed 100% prevalence of pSG3 and pSG4. Unlike isolates from other species of tsetse *S. glossinidius* GB required the haemolymph from 20 flies to initiate growth. The concentration of bacteria from so many individuals would increase the chance of the occurrence of *S. glossinidius* GB with pSG3 and pSG4 in the culture. This suggests that genes on these two extrachromosomal elements may be important for *in vitro* growth although further characterisations will be required to establish which genes may function in this process.

S.glossinidius prevalence showed no significant change over time, however, differences were found in *Wolbachia* spp. prevalences in wild *G. pallidipes* sampled one year apart. Although *Wolbachia* spp. has been implicated in causing cytoplasmic incompatibility (CI) in tsetse flies, no experimental evidence exists proving this, as antibiotic treatment of flies results in sterility. However, the current work suggests that CI may occur in tsetse as the prevalence of *Wolbachia* spp. in wild *G. pallidipes* sampled from Zimbabwe showed an increase from 0% to 80% over the course of a year. This could be due to the large scale migration into the area of *G. pallidipes* carrying *Wolbachia* spp. infections, however, it would be expected that large numbers of the original population would still be present were that the case. It is more likely that a smaller number of flies infected with *Wolbachia* spp. moved into the area and that this bacterium exerted a CI influence on the population, resulting in a replacement of the original population with *Wolbachia* spp. infected flies.

6.4 Conclusion

Nearly all studies into *S. glossinidius* have used bacteria isolated from laboratory colonies of tsetse. Given that the present work has shown that there is a high likelihood that this symbiont is potentially horizontally transmitted, both within and between species of tsetse, it may be that most of the work undertaken to date has used, what is effectively, a clonal strain of the same bacterium.

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